The Journal of LABORATORY and CLINICAL MEDICINE

VOL. 51 FEBRUARY, 1958 NO. 2

The Official Publication of the Central Society for Clinical Research

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Vol. 51, No. 2, February, 1958. The Journal of Laboratory and Clinical Medicine is published monthly by The C. V. Mosby Company, 3207 Washington Blvd., St. Louis 3, Mo. Subscription rates: United States and its Possessions $13.00, Students $6.50; Canada, Latin-America, and Spain $13.50, Students $7.00; Other Countries $14.50, Students $8.00. Single copies $2.00 postpaid. Entered as Second Class Matter October 8, 1915, at the Post Office at St. Louis, Missouri, under Act of March 3 1879. Printed in the U. S. A.

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Source—Free, H. M.; Cook, M. H., and Smeby, R. R.: Comparative Study of Qualitative Tests for Urine Ketones. Presented at 132nd National Meet., Am. Chem. Soc., New York, N. Y., Sept. 8-13, 1957. (Abstracts, p. 78 c).

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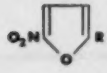

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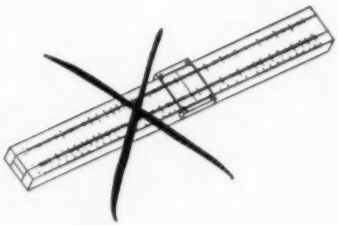

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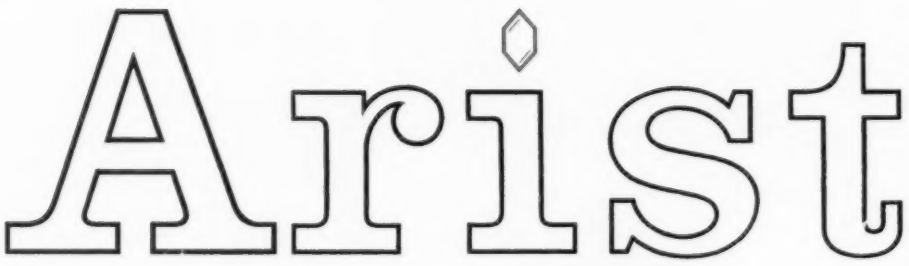

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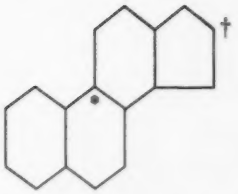

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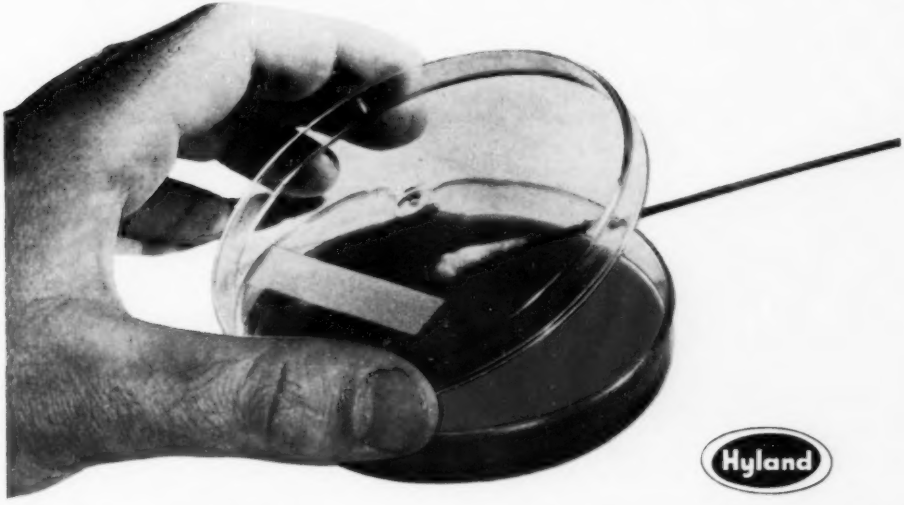

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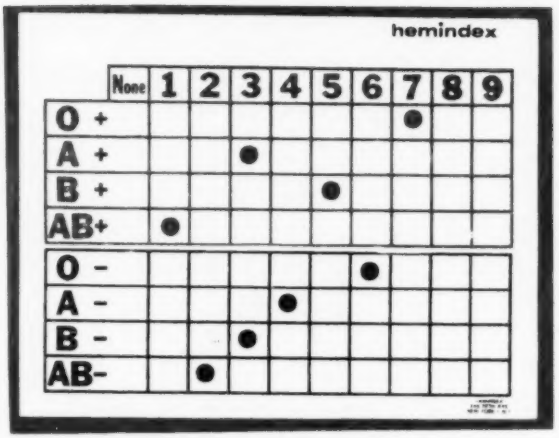

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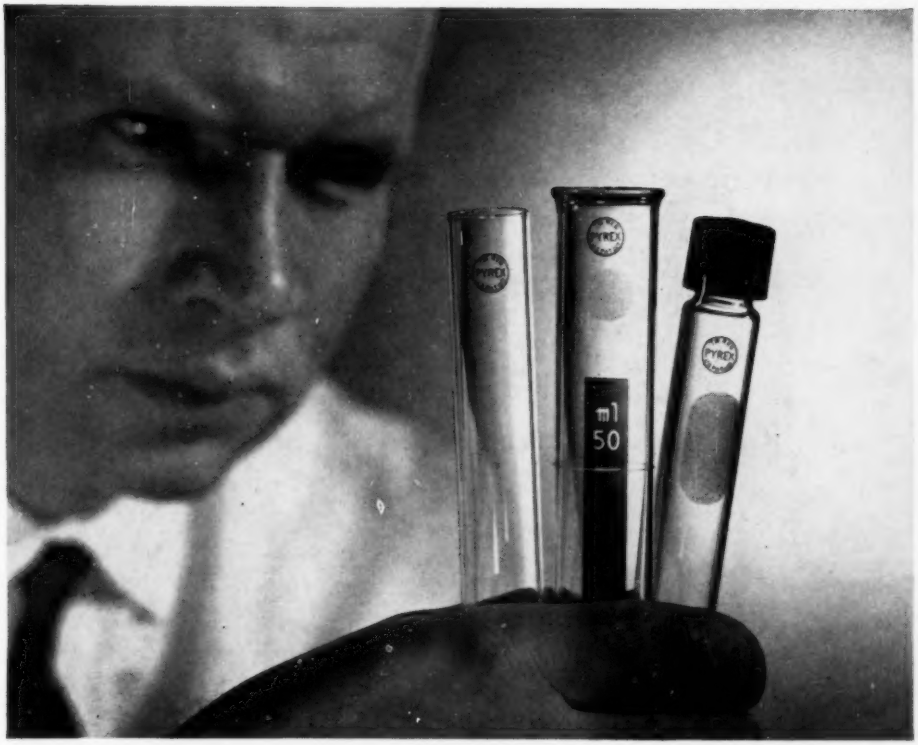

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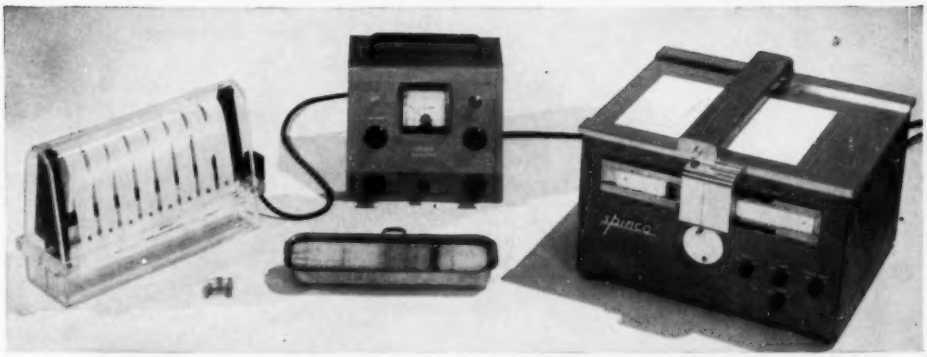

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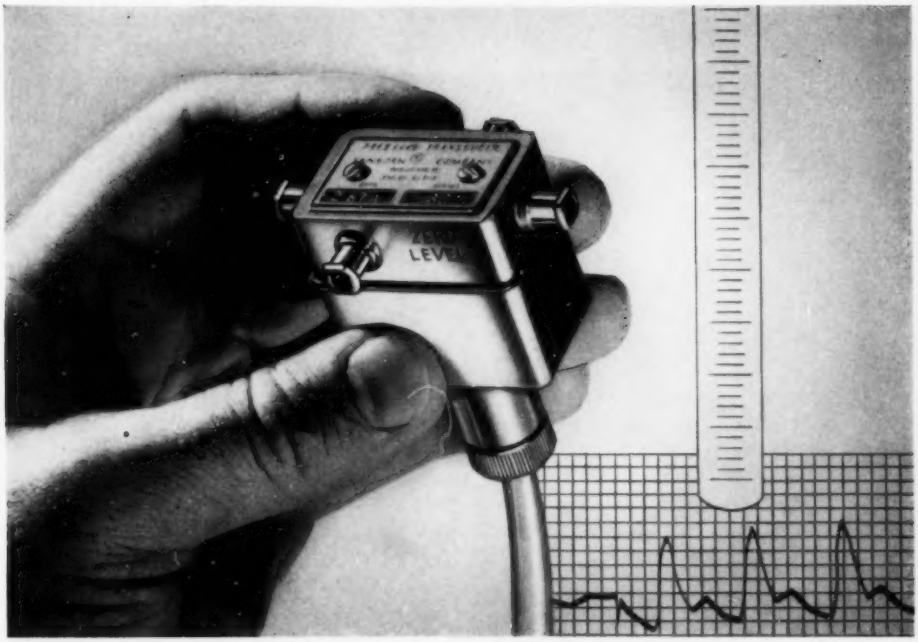

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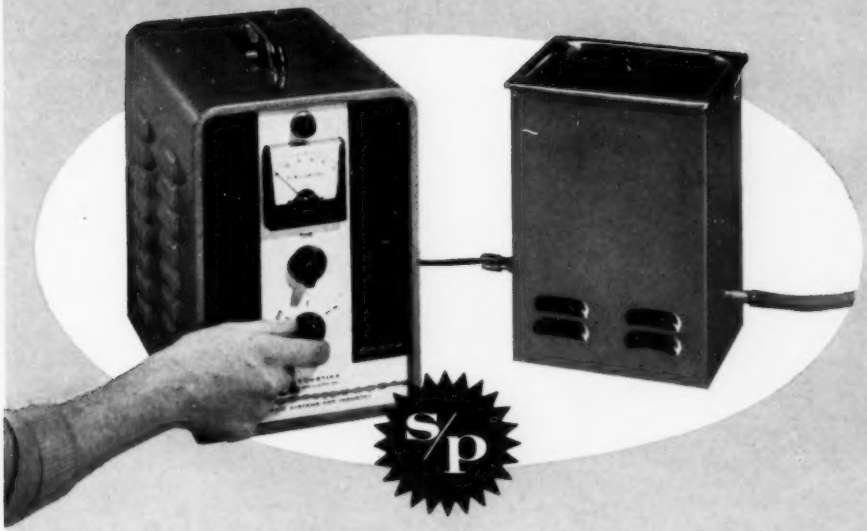

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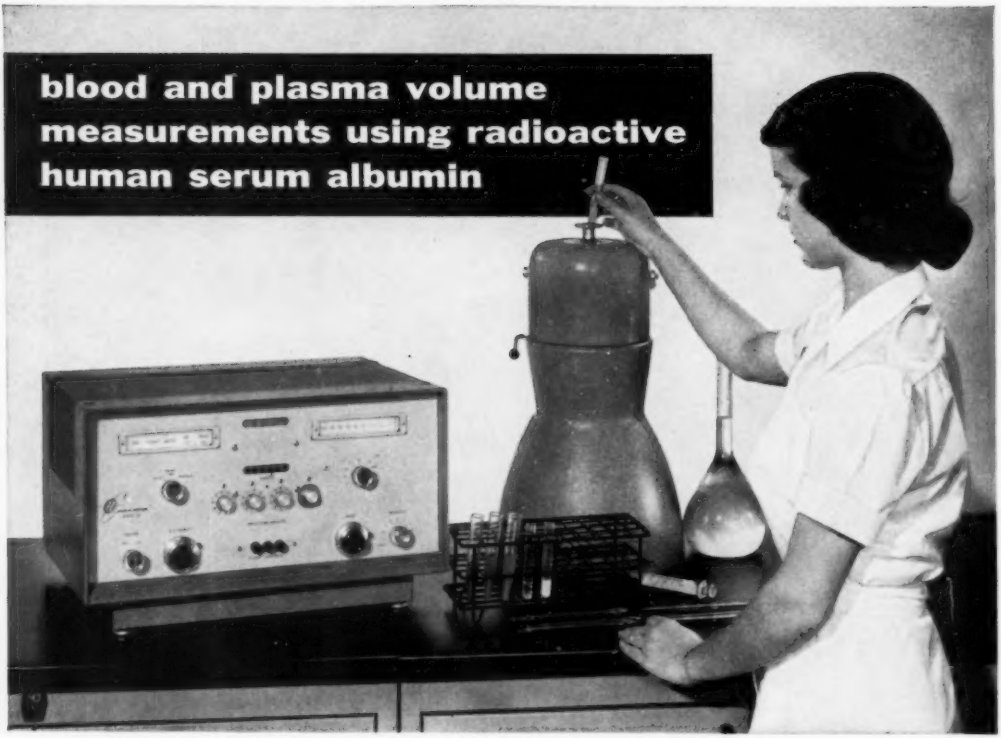

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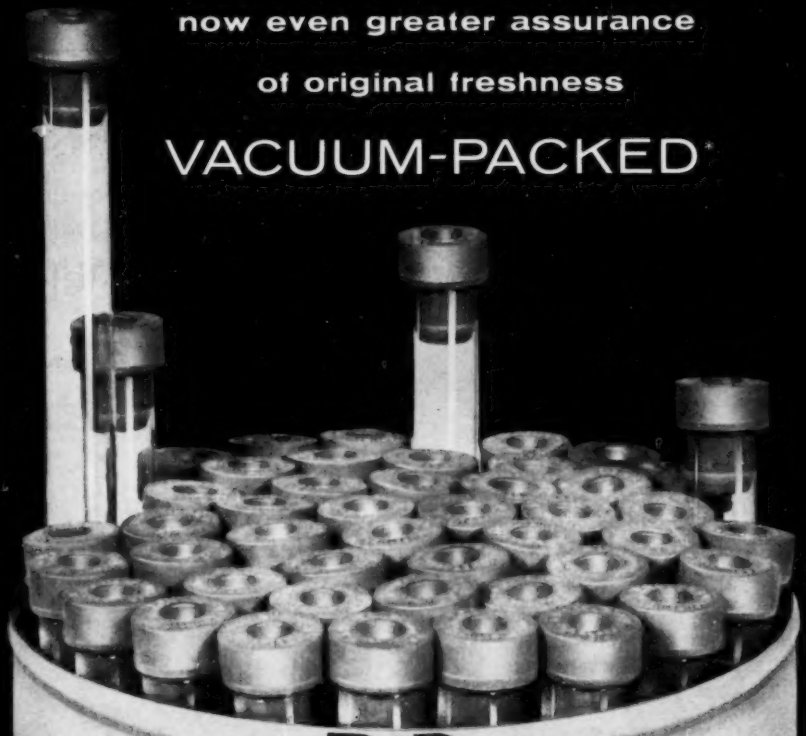

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CLINICAL AND EXPERIMENTAL

THE IMPORTANCE OF AMINO ACIDS AS DIALYZABLE COMPONENTS OF RAT SERUM WHICH PROMOTE SULFATE UPTAKE BY CARTILAGE FROM HYPOPHYSECTOMIZED RATS IN VITRO

WILLIAM D. SALMON, JR., M.D.,* AND WILLIAM H. DAUGHADAY, M.D.
ST. LOUIS, MO.

THE incorporation of sulfate by rat cartilage is profoundly influenced by the growth hormone of the anterior pituitary.[1-3] The great metabolic stability of cartilage under conditions of incubation in vitro has permitted us to examine the mechanism of hormonal regulation of this tissue. It was reported earlier[4] that the cartilage of hypophysectomized rats incorporated only about half as much sulfate as did cartilage from normal rats. Although injection of growth hormone into the hypophysectomized rat restored normal sulfate incorporation by the cartilage, the in vitro addition of growth hormone was virtually without effect on sulfate uptake by this tissue. However, when cartilage from hypophysectomized rats was incubated with serum from either normal or growth hormone-treated hypophysectomized rats, the incorporation of sulfate by this cartilage was greatly increased in comparison with that found with incubation in serum from hypophysectomized rats.

We also observed that some of the sulfation-promoting activity of serum from normal rats was lost by dialysis. This suggested either partial inactivation of a sulfation factor or the loss of dialyzable sulfation-promoting substance. The finding of Boström and co-workers[5, 6] that glutamine stimulates

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This work was supported by an Institutional Grant to Washington University from the American Cancer Society and by a research grant, A-1526, from the National Institute of Arthritis and Metabolic Diseases, National Institutes of Health.

Received for publication July 12, 1957.

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sulfate incorporation in vitro by beef cartilage suggested that loss of glutamine from serum by dialysis might have been responsible for the decline in sulfation activity. However, glutamine did not have a stimulating effect in our system on the sulfate incorporation by rat cartilage incubated either in a buffer medium alone or in a medium supplemented with dialyzed serum from normal rats.[4]

In this paper the sulfation-promoting activity of both nondialyzable and dialyzable components of serum from normal rats is reported. Evidence is also presented to suggest that a part of the dialyzable sulfation-promoting activity is attributable to essential amino acids.

METHODS

With the exceptions noted, the preparation and maintenance of rats, conditions of incubation, and analytical methods were essentially as described previously.[4] All experiments were of the random block design, costal cartilages from each hypophysectomized rat being assigned to each of the various treatment groups using a table of random numbers.[7] The basic medium is defined as Krebs' phosphosaline buffer (KPS)[8] to which the following were added in sufficient amounts to give the indicated concentrations per milliliter in the final medium: glucose 2 mg., penicillin 50 units, streptomycin 50 μg, and S^{35}-sulfate approximately 0.5 μc. Incubations were carried out at 37° C. for 24 hours. The inorganic sulfate of serum was determined by precipitating the sulfate with benzidine from a tricholoroacetic acid filtrate[9] and measuring the transmittance at 249 mμ of the solution of benzidine sulfate in 0.2 N hydrochloric acid, using a blank of the acid alone. All P values were calculated using the analysis of variance and the F. test.[10]

Preparation of Dialyzable and Nondialyzable Fractions of Serum From Normal Rats.—A volume of 15 ml. of serum from normal rats was dialyzed in a Visking cellophane bag for 48 hours at 4° C. against 150 ml. of redistilled water in a large, sealed glass tube on a revolving disk. The dialyzed serum and the dialysate were lyophilized. The lyophilized preparations were reconstituted so that they were isotonic in respect to sodium chloride and the sulfate concentration was 1.2 mM. The dialyzed serum was diluted to the protein concentration of the original serum. The dialysate residue was dissolved in a volume of 15 ml. The pH of all test solutions was adjusted to 7.4. The sulfate concentrations of the final media agreed within 0.5 per cent, so that corrections for differences in the sulfate pool were minimal.

Preparation of Amino Acid and Vitamin Solution.—The amino acids and vitamins were those found by Eagle and co-workers[11] to be essential for tissue culture of HeLa cells, plus inositol, which the same investigators[12] also found essential for a number of normal and malignant human cells in tissue culture. For assay purposes the amino acids were divided into 6 groups: basic (arginine, histidine, lysine); sulfur-containing (cystine, methionine); heterocyclic (tryptophane); aromatic (phenylalanine, tyrosine); aliphatic, monoaminomonocarboxylic (isoleucine, leucine, threonine, valine); and monoaminodicarboxylic (the amide, glutamine). L-Amino acids* were prepared in the following concentrations, in millimoles per liter, in Krebs' phosphosaline buffer: arginine 0.1, histidine, 0.05, lysine 0.2, cystine 0.05, methionine 0.05, tryptophane 0.02, phenylalanine 0.1, tyrosine 0.1, isoleucine 0.2, leucine 0.2, threonine 0.2, valine 0.2, and glutamine 2.0. The vitamins* (D-biotin, choline, folic acid, I-inositol, nicotinamide, calcium D-pantothenate, pyridoxal hydrochloride, riboflavin, and thiamine hydrochloride) were prepared in concentrations of 1 mcg. per milliliter, except for riboflavin in a concentration of 0.1 mcg. per milliliter, in Krebs' phosphosaline buffer.

RESULTS

The sulfation-promoting activity lost by dialysis of serum from normal rats was recovered in the dialysate (Table I). The dialysate had activity similar to that of the dialyzed serum in stimulating sulfate incorporation by costal

*All amino acids and vitamins were obtained from Nutritional Biochemicals Corporation, Cleveland, Ohio, except D-biotin which was obtained from Hoffmann-La Roche, Inc., Nutley, N. J.

cartilage from hypophysectomized rats. The activity of the original serum was restored by combining the dialyzed serum and the dialysate, and the combined effect was more than additive ($P<0.001$), indicating interaction between the fractions.

The nature of the dialyzable sulfation-promoting component was sought by testing the effects of known constituents of serum on sulfate incorporation by cartilage from hypophysectomized rats. The addition to the incubation medium of 13 amino acids increased sulfate uptake by the cartilage (Table II).

TABLE I. EFFECTS OF DIALYZABLE AND NONDIALYZABLE COMPONENTS OF SERUM FROM NORMAL RATS ON SULFATE INCORPORATION IN VITRO BY COSTAL CARTILAGE FROM HYPOPHYSECTOMIZED RATS

GROUP	TEST SUPPLEMENT	ML.	SULFATE UPTAKE (μG/100 MG. WET CARTILAGE) (MEAN ± S.E.)
A	None	- -	7.8 ± 0.5
B	Normal rat serum	0.1	20.0 ± 0.7
C	Dialyzed serum	0.1	12.7 ± 0.6
D	Dialysate	0.1	12.9 ± 0.8
E	Dialyzed serum plus dialysate	0.1 0.1	22.1 ± 0.7

Conditions of Incubation: Each group contained one costal cartilage from each of 9 hypophysectomized rats. Each cartilage sample was incubated in 0.5 ml. of basic medium plus a test supplement made up to 0.2 ml. with isotonic salt solution where indicated to give a final volume of 0.7 ml.

TABLE II. EFFECTS OF ALIPHATIC, MONOAMINOMONOCARBOXYLIC AMINO ACIDS, ALONE AND TOGETHER WITH OTHER ESSENTIAL AMINO ACIDS AND VITAMINS, ON SULFATE INCORPORATION BY COSTAL CARTILAGE FROM HYPOPHYSECTOMIZED RATS

GROUP	ADDITIONS	S^{35}-SULFATE UPTAKE (C.P.M./MG. WET CARTILAGE) MEAN ± S.E.	P VALUE
A	None	44.6 ± 3.5	B-A <0.01
B	Isoleucine, leucine, threonine, valine	59.8 ± 4.3	C-A <0.001
C	Eagle's amino acids	72.7 ± 5.3	C-B <0.01
D	Eagle's amino acids and vitamins	78.7 ± 6.2	D-C <0.2

Conditions of Incubation: Each assay group contained one costal cartilage from each of 8 hypophysectomized rats. Each cartilage sample was incubated in 0.3 ml. basic medium plus 0.4 ml. KPS buffer which, in the designated experiments, contained amino acids and vitamins in the concentrations described under Methods.

This effect was not significantly augmented by adding the vitamin supplement, and the vitamins alone did not increase sulfate incorporation. In a number of experiments the amino acids increased sulfate uptake by cartilage from hypophysectomized rats between 50 and 120 per cent. When the separate effects of 6 groups of the amino acids were studied, it was found that only the aliphatic, monoaminomonocarboxylic amino acids (isoleucine, leucine, threonine, and valine) stimulated sulfate incorporation. However, this stimulation was only one-third to one-half that of the total amino acid mixture. Threonine produced the highest mean sulfate uptake of any one of these four amino acids tested individually, but the stimulation was not statistically significant.

Omitting any one of the 6 groups of amino acids from a medium containing the others resulted in lower sulfation activity than when all were present (Table III, Experiment 1), although the lowering from the omission of the

basic amino acids (arginine, histidine, and lysine) was not significant. There was no stimulation of sulfate incorporation by cartilage from hypophysectomized rats in the absence of the aliphatic, monoaminomonocarboxylic amino acids. When either isoleucine or leucine alone was omitted from the medium,

TABLE III. EFFECT ON SULFATION-PROMOTING ACTIVITY OF OMITTING CERTAIN AMINO ACIDS FROM EAGLE'S AMINO ACID MIXTURE

EXPERIMENT	GROUP	AMINO ACIDS OMITTED	S^{35}-SULFATE UPTAKE (C.P.M./MG. WET CARTILAGE) MEAN ± S.E.	P VALUE
1	A	All	38.1 ± 3.6	
	B	None	74.8 ± 6.6	B-A <0.001
	C	Arginine, histidine, lysine	71.4 ± 6.8	B-C <0.3
	D	Glutamine	67.2 ± 5.6	B-D <0.05
	E	Phenylalanine, tyrosine	57.0 ± 5.7	G-A <0.001
	F	Cystine, methionine	52.4 ± 4.2	A-H <0.2
	G	Tryptophane	52.4 ± 4.6	
	H	Isoleucine, leucine, threonine, valine	33.9 ± 3.2	
2	A	All	29.2 ± 4.2	B-A <0.001
	B	None	60.5 ± 10.1	D-A <0.01
	C	Isoleucine	42.8 ± 6.9	B-C <0.001
	D	Leucine	42.4 ± 7.9	A-F <0.05
	E	Valine	30.0 ± 6.4	
	F	Threonine	20.0 ± 3.1	

Conditions of Incubation: In both experiments, each assay group contained one costal cartilage from each of 8 hypophysectomized rats. Each cartilage sample was incubated in 0.2 ml. basic medium plus 0.5 ml. KPS buffer containing, with the omissions as indicated, amino acids in the concentrations described under Methods.

TABLE IV. EFFECT ON SULFATION-PROMOTING ACTIVITY OF ADDING AMINO ACIDS TO SERUM FROM HYPOPHYSECTOMIZED AND NORMAL RATS, BEFORE AND AFTER DIALYSIS OF THE SERUM

GROUP	ADDITIONS	SULFATE UPTAKE (μG/100 MG. WET CARTILAGE) MEAN ± S.E.	P VALUE
	Experiment 1. Serum from hypophysectomized rats		
A	None	8.8 ± 1.1	B-A <0.001
B	Amino acids	12.8 ± 1.3	C-A <0.001
C	Serum	11.3 ± 0.7	D-C <0.001
D	Serum plus amino acids	13.6 ± 1.1	D-B <0.2
E	Dialyzed serum	9.0 ± 0.6	F-E <0.001
F	Dialyzed serum plus amino acids	12.1 ± 0.9	B-F <0.3
			D-F <0.01
	Experiment 2. Serum from normal rats		
A	None	9.5 ± 0.7	B-A <0.001
B	Amino acids	15.4 ± 1.1	C-A <0.001
C	Serum	21.4 ± 0.7	D-C <0.001
D	Serum plus amino acids	34.8 ± 2.1	D-B <0.001
E	Dialyzed serum	15.2 ± 1.2	F-E <0.001
			F-B <0.001
F	Dialyzed serum plus amino acids	24.8 ± 1.3	D-F <0.001

Conditions of Incubation: In both experiments, each assay group contained one costal cartilage from each of 8 hypophysectomized rats. Each cartilage sample was incubated in 0.2 ml. basic medium plus 0.1 ml. serum or KPS buffer plus 0.4 ml. KPS buffer containing Eagle's amino acids in the concentrations described under Methods.

a significant stimulating effect of the other amino acids remained (Table III, Experiment 2). However, the absence of valine abolished the stimulation from the other amino acids and the absence of threonine resulted in a medium in which significantly less sulfate was incorporated than in the KPS buffer.

The sulfation-promoting effects of amino acids were also measured in media containing either normal or dialyzed rat serum (Table IV). When

amino acids were added to serum from hypophysectomized rats, either before or after dialysis of the serum, the effect on sulfate incorporation by cartilage from hypophysectomized rats was not significantly different from that obtained by adding amino acids to a buffer medium without serum. Adding amino acids to media containing serum from normal rats, both before and after dialysis of the serum, increased sulfate incorporation by cartilage incubated in these media in proportion to the levels of sulfate uptake attained with incubation in media without added amino acids. The augmentation of sulfate uptake by the addition of amino acids in this experiment, was 63 per cent in the case of the buffer medium and 62 per cent in the media containing normal or dialyzed serum from normal rats. Adding amino acids to dialyzed serum from either normal or hypophysectomized rats did not restore sulfate incorporation to the levels obtained when amino acids were added to the respective normal sera. In other experiments, not shown, sulfation was not increased further by adding the vitamin supplement to the dialyzed serum from normal rats plus the amino acids, even with careful attention to possible alterations in protein concentration resulting from the dialysis.

DISCUSSION

These experiments demonstrate the presence of both nondialyzable and dialyzable sulfation-promoting components of serum from normal rats. The nondialyzable component, probably protein in nature or protein-bound, appears to be dependent on pituitary function, since it has not been found in the serum of rats or humans[13] after hypophysectomy. The specificity of the nondialyzable component in this in vitro incubation system is supported by the finding that the albumin fraction of serum from normal rats (prepared by continuous flow, paper electrophoresis) is inactive.* Whether or not the nondialyzable sulfation-promoting component of serum from normal rats acts in our system in a manner analogous to that of the protein factor of serum required for prolonged survival and growth of other cells in tissue culture[14] is not known. However, Jacquez and Barry[15] have reported that serum from hypophysectomized rats served as well as serum from normal rats for growth of rat fibroblasts in tissue culture.

The nature of the dialyzable sulfation-promoting component, found to some extent also in serum from hypophysectomized rats, is at least suggested by the experimental results. A mixture of amino acids found essential for growth of certain cells in tissue culture stimulates sulfate incorporation by cartilage from hypophysectomized rats. This effect cannot be attributed to any single amino acid. Omitting various amino acids from the mixture decreases the sulfation-promoting activity. Threonine appears to be the limiting amino acid, and absence of threonine from the mixture results in a medium with sulfation-inhibiting properties. Valine seems to be second in importance. The absence of other amino acids results in a medium with varying degrees of sulfation-promoting activity. The reason for the sulfation-promoting effect of amino acids is not apparent. The multiple dependence on amino acids and the

*Unpublished experiments.

clear-cut demonstration of amino acid imbalance effects suggests that protein synthesis is necessary for increased rates of sulfation and that the amino acids are not acting as donors for amino groups or as energy sources.

The sulfation-promoting effects of these essential amino acids and dialyzed serum from normal rats are more than additive and, in this respect, are similar to the effects of serum dialysate and dialyzed serum. An interaction between the nondialyzable sulfation-promoting component of serum and essential amino acids is suggested. The former, the hormonally dependent sulfation factor, may act to promote amino acid uptake and protein synthesis in cartilage, an action consistent with the known effect of growth hormone in vivo.[16-19]

Other dialyzable sulfation-promoting substances may be present in serum, because the addition of amino acids to dialyzed serum from either normal or hypophysectomied rats did not restore the level of sulfation activity to that obtained when the same concentrations of amino acids were added to the respective normal sera. When one compares the concentrations of these free amino acids reported found in rat serum[20, 21] with the concentrations added in these experiments, it seems unlikely that these differences in sulfation activity are due to the greater content of these same amino acids in the media containing serum not dialyzed. The presence of amino acids or vitamins other than those added in these experiments may be of importance. Metal constituents do not contribute to the sulfation-promoting effect of serum from normal rats, since this activity is unaffected by the addition of chelating agents, such as the sodium salt of ethylenediaminetetraacetic acid or sodium citrate, and, furthermore, is completely destroyed by ashing.* Glucosamine,† found by Roden[22] to stimulate sulfate uptake by beef cartilage in vitro, had no effect in our system on sulfate incorporation by cartilage from hypophysectomized rats.*

SUMMARY

1. Serum from normal rats contains both nondialyzable and dialyzable components which stimulate sulfate incorporation in vitro by cartilage from hypophysectomized rats. Serum from hypophysectomized rats does not contain the nondialyzable component, indicating its dependence on the function of the pituitary gland.

2. A mixture of thirteen L-amino acids has a stimulating effect on sulfate incorporation by cartilage from hypophysectomized rats. This effect is not attributable to any single amino acid. Of 6 groups of these amino acids assayed, only the aliphatic, monoaminomonocarboxylic amino acids (isoleucine, leucine, threonine, and valine) increased sulfate uptake by the cartilage. This increase was only one-third to one-half that produced by the total amino acid mixture. Threonine appears to be the limiting amino acid in these 13, with valine second in importance.

3. Addition of the amino acids to a medium containing serum from hypophysectomized rats did not significantly increase sulfate incorporation in vitro by cartilage from hypophysectomized rats over the level attained by cartilage incubated in a buffer medium with these amino acids. Addition of the amino acids to a medium containing either normal or dialyzed serum from normal

*Unpublished experiments.

†D(+)-Glucosamine hydrochloride: obtained from Nutritional Biochemicals Corporation, Cleveland, Ohio.

rats increased sulfate incorporation in vitro by cartilage from hypophysectomized rats in proportion to the uptake attained by cartilage incubated in similar media without added amino acids.

4. The addition of 9 vitamins to a buffer medium or to media containing amino acids, with or without serum, did not significantly increase sulfate uptake by cartilage incubated in these media.

5. It is suggested that a part of the dialyzable sulfation-promoting activity of serum is attributable to amino acids. An interaction between the non-dialyzable, probably protein or protein-bound, component and amino acids is also suggested.

We are indebted to Miss Marilyn Mitchell for technical assistance.

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THE EFFECT OF HYDROCORTISONE AND CORTISONE ON FIXATION OF S^{35} IN THE STOMACH

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THE occurrence of ulcers in the gastrointestinal tract of patients receiving cortisone and hydrocortisone has led to many studies seeking to explain this phenomenon. The experimental reports have been somewhat in conflict. Welbourn and Code[1] found no evidence that cortisone acetate affects the incidence of ulceration of the stomach in rats. On the other hand, adrenal steroids are reported to produce increased gastric secretion of acid and pepsin thereby inducing ulcers.[2] Ingle and associates[3] state that large doses of cortisone administered over a long period may increase the incidence of ulceration in the glandular portion of the stomach of the rat. Histologic studies by Baker[4] indicate that adrenocorticotrophin reduces the thickness of the gastric mucosa in intact rats thereby decreasing the protective mechanism against the action of hydrochloric acid. In hypophysectomized rats, the administration of cortisone and hydrocortisone tends to restore the structure and function of the atrophic gastric chief cells.[5]

Studies utilizing tracer doses of S^{35} as $Na_2S^{35}O_4$ have shown that radiosulfur is readily fixed by the mucus-secreting glands of the stomach.[6, 7] In general the connective tissue actively incorporates S^{35} into sulfated compounds such as chondroitin sulfate, a polysaccharide present in mucin as well as other portions of the connective tissue system.[8-10] This paper deals with the effect of cortisone acetate and hydrocortisone acetate on the fixation of radiosulfur, S^{35}, in the stomach of hypophysectomized rats.

METHODS

Young female rats served as experimental animals. They were hypophysectomized at 23 days of age and used experimentally on the sixth postoperative day. Hypophysectomized rats were used since they were found to be more sensitive to the action of cortisone and hydrocortisone than were normal animals.† Determining completeness of removal of the hypophysis was a difficult problem that could be resolved by several approaches. Serial section and microscopic study of each pituitary region was not feasible. Several animals were examined grossly at autopsy and no tissue was found in the pituitary region. Animals were weighed carefully each day to note absence of weight gain. Those which gained more than 2 or 3 grams during the observation and experimental period were not used in this study. However, it is possible that an occasional animal with residual hypophyseal tissue

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may have been inadvertently included despite the precautions taken. Animals were litter mates and all weighed approximately the same, 55 to 60 grams at the start. All animals that had evidence of respiratory infections were eliminated.

Animals were kept in an air-conditioned laboratory at a constant temperature. Since hypophysectomized rats would not readily eat the usual laboratory diet, they were fed daily an optimum diet, a special mixture of canned dog food, milk, and bread ground in a Waring blendor. Daily supplements of carrot and lettuce were given to maintain optimal nutrition. Food and tap water were consumed ad libitum.

Hydrocortisone acetate was administered for 4 days intraperitoneally in doses of 25, 50, and 100 μg each in 0.5 ml. of physiologic saline. Cortisone acetate was given for 4 days in doses of 2.5 mg. in 0.1 ml. of saline. Saline was administered to control animals in doses equal in volume to that of the hormone injection. Radiosulfur as $Na_2S^{35}O_4$ in doses of 10 μc was administered concurrently with the hormone or saline. Groups of 4 to 5 animals were used at each dosage level. Experiments were repeated a minimum of 3 times.

Animals were killed by etherization 24 hours after the last dose of hormone and S^{35}. The stomachs were removed, cleaned, observed carefully for ulceration then separated into fore-stomach and glandular stomach before being assayed for radioactivity. Details of the procedure in treating samples have been presented previously.[11] Occasionally tissues were frozen for a few days before processing. The radioactivity was determined to a probable error of 3 to 5 per cent in a windowless gas flow counter. Correction was made for radiodecay. The radiosulfur, S^{35}, was obtained from Oak Ridge National Laboratories, Oak Ridge, Tenn.

TABLE I. THE EFFECT OF CORTISONE AND HYDROCORTISONE ON FIXATION OF S^{35} IN THE STOMACH

SITE	NO. ANIMALS	C.P.M. PER 100 MG.* MEAN	±	S.E.	RANGE	SIGNIFICANCE OF COMPARISON
Fore-stomach						
Control	7	312	±	65	196- 480	
Hydrocortisone†	16	237	±	78	114- 380	$P = < 0.05$
Glandular stomach						
Control	7	183	±	37	130- 240	
Hydrocortisone	16	110	±	25	80- 160	$P = < 0.01$
Fore-stomach						
Control	9	2,260	±	600	1,050-3,140	
Cortisone†	6	1,080	±	160	865-1,350	$P = < 0.01$
Glandular stomach						
Control	9	1,355	±	405	900-2,350	
Cortisone	6	590	±	255	310- 950	$P = < 0.01$

*c.p.m. per 100 mg. = counts per minute per 100 mg. of wet tissue.

†Dose of hydrocortisone acetate was 25 to 100 μg; dose of cortisone acetate was 2,500 μg.

RESULTS AND DISCUSSION

Hydrocortisone acetate and cortisone acetate produced a significant reduction in the fixation of S^{35} in the stomach of the hypophysectomized rat (Table I). Approximately 15 per cent of the animals receiving these steroids had small ulcers in the glandular stomach where others have reported them to occur following this type of treatment. Since the inhibition of S^{35} uptake by hydrocortisone was similar at each of the dosage levels, the hydrocortisone-treated animals were considered as a group. It is possible that some of this variation may be due to remnants of hypophyseal tissue. The dosage of cortisone acetate, 2.5 mg. per day in rats weighing 55 or 60 grams, is admittedly

a very high one when related to the dosage used clinically in human patients. At this dose range the effect of cortisone may be a pharmacologic one. Nonetheless, these results indicate that the mechanism of ulcer formation and decreased fixation of S^{35} do occur in rats receiving high doses of cortisone.

The dosage of hydrocortisone acetate, 25 to 100 μg given to rats weighing 55 to 60 grams, is more closely related to clinical usage. These figures correspond fairly well with our knowledge of hydrocortisone production in human subjects. Peterson and Wyngaarden[12] have reported that their normal subjects produced from 16 to 26 mg. of hydrocortisone daily while the total endogenous hydrocortisone production was found to be 181 mg. in a subject receiving maximal corticotrophin stimulation.

Attempts to explain the ulceration in the gastrointestinal tract of steroid-treated animals and patients should consider the parts played by the protective action of mucin and the tissue resistance of the normal connective tissue found in this area. Hirschowitz and co-workers[13] have reported a decrease in the amount and viscosity of mucus in the gastric juice of human subjects receiving corticotrophin. The decreased S^{35} incorporation into the stomach presumably reflects a decreased synthesis of the sulfated compounds such as chondroitin sulfate in the mucin and in the ground substance of the connective tissue of the gastrointestinal tract. Cortisone and hydrocortisone have been reported to inhibit the synthesis of chondroitin sulfate in the skin of the rat.[14]

These steroids may exert their effect on S^{35} fixation by inhibiting the enzymatic transfer of sulfate into the sulfated compounds occurring in the gastrointestinal tract. Perhaps these drugs, well known as antianabolic or catabolic agents, may decrease production of enzymes or factors that are vital in sulfate transport. Other components of the sulfated-mucopolysaccharide molecule may be adversely affected. A retardation in the healing of ulcerated areas may also be produced by this inhibitory action of the steroids on the connective tissue. The influence of cortisone and hydrocortisone in reducing the protective action of mucin and in inhibiting the elements maintaining normally the connective tissue of the gastrointestinal tract should be considered as significant factors in the etiology of ulcers in patients receiving these steroids.

SUMMARY

Administration of hydrocortisone acetate and cortisone acetate to hypophysectomized rats reduced the incorporation of S^{35} into the tissue of the stomach by approximately one-third to one-half of the values noted in control animals. The dose of hydrocortisone used, 25 to 100 μg per day, corresponds to levels used clinically in human subjects, while the dose of cortisone acetate, 2,500 μg, exceeds that in usual clinical practice. This influence of the steroid hormones is discussed as a significant factor in the etiology of ulcers by reducing the formation and the protective action of the mucin and by inhibiting factors which normally maintain connective tissue.

I am grateful for the excellent technical assistance of Martin Nettleship and Thomas Pape in the course of these experiments.

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MECHANISMS REGULATING THE RETENTION OF SODIUM IN THE FECES BY CATION-EXCHANGE RESIN: RELEASE OF BASE FROM THE RESIN BY BACTERIAL FERMENTATION IN THE TERMINAL ILEUM

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THE clinical use of cation-exchange resins has largely been abandoned despite the generally favorable reports of their effectiveness in the treatment of edema. To be sure, the need for some immediate precautions was recognized. Also, some reservations were made concerning possible deleterious effects which might result from their prolonged use.[1]

The mechanisms by which cation-exchange resins retain much less sodium in the feces during the consumption of low-sodium diets than when the sodium intake was less restricted[2-6] have been investigated. A carboxylic cation-exchange resin, Resodec, was equilibrated with a solution containing the average concentrations of electrolytes found by Lockwood and Randall[7] in the dejecta from recent ileostomies. It was observed that the feces of patients with recently compensated heart disease, who were maintained on diets with 800 to 1,200 mg. of sodium per day and who received that resin for 4- to 7-week periods, contained an average of one-fifth as much sodium and 8 times as much potassium, per gram of resin, as was bound by the resin in that equilibrium.[8]

McCance[9] reported decreased concentrations of sodium in the saliva and gastric juice during sodium depletion resulting from restriction of dietary sodium and diuresis. We investigated further the conservation of sodium in the upper gastrointestinal tract, using dogs with fistulas into the terminal ileum. A severe restriction of dietary sodium was followed by a transition from high-sodium and low-potassium concentrations, in the contents of the terminal ileum, to the reverse, i.e., low-sodium and high-potassium concentrations.[10] By feeding the same bread at 4 different levels of sodium content, alone and with the accompanying administration of cation-exchange resin, it was shown that the changes in sodium and potassium concentrations in the terminal ileum (sodium conservation), were proportional to the degree of restriction of dietary sodium.[11]

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This work was supported in part by research grants from the National Heart Institute (H-1970), United States Public Health Service, and from the American Heart Association.

The cation-exchange resin used in these experiments was generously supplied by Smith, Kline & French Laboratories, Philadelphia, Pa.

Received for publication July 16, 1957.

Despite the considerable conservation of sodium in the upper gastrointestinal tract there was, during the administration of the resin to those dogs, at all levels of restriction of sodium intake, much more sodium bound by the resin, in the terminal ileum, than was provided by the diet. The colon absorbed a major amount of the sodium presented to it. Yet there was more sodium in the feces than was provided by the diet. That could be true because during the administration of resin and restriction of dietary sodium there was, in the terminal ileum, more sodium and potassium free in solution than was bound by the resin.[11]

The high concentrations of unbound sodium and potassium in the terminal ileum, under those circumstances, was a notable finding in those experiments. It was suggested that sodium and potassium were being released from the resin, in the terminal ileum, faster than they were being absorbed.[11] It is the purpose of this paper to show that those ions were being released from the resin by hydrogen ions provided by fermentation in the terminal ileum; also to remark upon the felicitous circumstance that the mechanisms outlined above were operating. Otherwise, the uninhibited withdrawal of sodium from the body could have been calamitous.

MATERIAL AND METHODS

In these experiments and those previously reported, the carboxylic cation-exchange resin, Resodec, which is 22 per cent in the potassium cycle and 78 per cent in the ammonium cycle, was used. The preparation of fistulas into the small intestine of dogs has been described.[10] The methods for the determination of free and resin-bound sodium and potassium were as previously described.[11]

RESULTS

It seemed probable that hydrogen ions were the only ions that would be likely to displace sodium and potassium from the resin, in the terminal ileum. Bacterial fermentation seemed to be the probable source of hydrogen ions. Indirect evidence supporting those assumptions was obtained by administering unabsorbable antibiotics. During a 7-day control period of diet plus Resodec, two ileal fistula dogs had averages of 165 and 164 mEq., respectively, of unbound sodium per liter in the contents of the terminal ileum. During 5 subsequent days, on the same regimen plus the administration of 0.5 Gm. each of neomycin and bacitracin 3 times a day, the free sodium in the ileal contents of those dogs averaged 143 and 135 mEq. per liter, respectively. Each day's value for the unbound sodium, from which the above averages were calculated, was the result of the analysis of a pool of equal amounts of 4 or 5 samples collected at hourly intervals, beginning 1½ to 2 hours after feeding.

Untreated ileal fluid from other fistula dogs was found to release sodium and potassium from the resin while an autoclaved aliquot of the same fluid did not do so. But the ileal fluid from those dogs might have been contaminated during previous samplings by catheter aspiration. Consequently, dogs were sacrificed to obtain the naturally occurring contents of the terminal ileum.

TABLE I. THE EFFECT OF AUTOCLAVING ON ELECTROLYTE DISTRIBUTION WHEN PARTIALLY SATURATED CATION-EXCHANGE RESIN WAS ADDED TO FRESH CONTENTS OF THE TERMINAL ILEUM OF DOGS

	NOT AUTOCLAVED					AUTOCLAVED BEFORE ADDING RESIN				
		SODIUM		POTASSIUM			SODIUM		POTASSIUM	
	pH	FREE (mEq./L.)	BOUND (mEq./GM.)	FREE (mEq./L.)	BOUND (mEq./GM.)	pH	FREE (mEq./L.)	BOUND (mEq./GM.)	FREE (mEq./L.)	BOUND (mEq./GM.)
Preresin	6.6	58		14		6.0*	58		17	
5 min. after resin	7.0	136	3.0	15	0.41	6.4	134	2.3	20	0.39
1 hr. after resin	6.8	157	2.8	16	0.34	6.6	138	2.2	20	0.37
2 hr. after resin	6.6	172	2.7	20	0.38	6.6	136	2.3	20	0.38
4 hr. after resin	6.5	206	1.8	24	0.24	6.5	136	2.2	20	0.38

*This difference from the pH of the untreated aliquot of the same specimen is the result of autoclaving. The resin was added to each aliquot after the autoclaving.

Samples of that fluid were divided into two parts. One part was autoclaved. Then, equal amounts of the cation-exchange resin, which had previously been partially saturated with sodium and potassium, at approximately the pH of the ileal fluid being tested, were added to each part of the fluid. At 5 minutes, there was a considerable transfer, by exchange, of sodium and potassium from the resin to the solution in both specimens (Table I). Subsequently, as the two specimens stood at room temperature for two hours, there were considerable further transfers of sodium and potassium from the resin to the solution, in the unautoclaved fraction. No such changes, in either the free or the resin-bound sodium and potassium, occurred in the autoclaved specimen.

In order to obtain a more complete depiction of transfers of sodium and potassium onto and off the resin, in the gastrointestinal tract, dogs were sacrificed 2½ hours after a normal feeding of canned dog food and bread and the subsequent administration, by stomach tube, of 15 Gm. of Resodec. That time was chosen because it was then that, in our fistula dogs, adequate amounts of resin had arrived at the terminal ileum and were still present in the jejunum. The small intestine was divided into 6 segments. The first 5 segments, beginning with the duodenum were 12 to 18 inches long. The last segment (number 6) was the last 6 inches of the terminal ileum. The free and the resin-bound sodium and potassium and the pH of the contents of each segment are shown in Table II. It is apparent, as expected, that the resin had been completely, or almost completely, converted into its hydrogen cycle by the HCl of the stomach. Also, it is probable that there had been a delay in emptying of the stomach, followed by a rapid passage through the intestine. Other similarly treated dogs had had larger amounts of sodium and potassium bound by the resin in the middle portion of the small intestine.

TABLE II. ELECTROLYTE DISTRIBUTION IN SUCCESSIVE SEGMENTS OF THE SMALL BOWEL OF A DOG 2½ HOURS AFTER A FEEDING AND SUBSEQUENT ADMINISTRATION OF CATION-EXCHANGE RESIN

SEGMENT NUMBER	pH	SODIUM		POTASSIUM	
		FREE (mEq./L.)	BOUND (mEq./GM.)	FREE (mEq./L.)	BOUND (mEq./GM.)
1	5.7	90	0.08	36	0.01
2	5.8	103	0.18	29	0.04
3	6.4	122	0.30	29	0.07
4	6.6	146	0.83	19	0.13
5	7.3	152	4.2	18	0.38
6		166	3.2	20	0.39

The large amount of potassium in the first segments of the small bowel is presumed to be from the meat of the diet. In the last segment, there was an increase in the free sodium to a considerable hypertonicity of the combined ions while the sodium bound by the resin, which had been 4.2 mEq. per gram in the preceding segment, declined to 3.2 mEq. per gram of resin.

DISCUSSION

These experiments, together with those previously reported, provide explanations for the limitations imposed on the amounts of sodium that can be retained in the feces, by an effective and usable cation-exchange resin, during restriction of dietary sodium. The body usually conserves sodium very effectively. Danowski and his associates[12] found that the administration of a cation-exchange resin did not significantly increase the stool losses of sodium in nephrotic children who were maintained on an essentially sodium-free regimen.

Spencer, Ross, and Lloyd-Thomas[13] have reported findings similar to part of our findings. They obtained specimens from patients on a low-salt diet and cation-exchange resin, by Miller-Abbott tube, from a fresh ileostomy or at autopsy, after sudden death. They found progressively decreasing amounts of sodium and increasing amounts of potassium, bound by the resin, in the small intestine, proximal colon, and distal colon, respectively. They assumed that dietary salt restriction did not materially alter the sodium content of the fluid in the small intestine. When they introduced the resin into isolated loops of the small intestine of rats, they found a large uptake of sodium, progressive over 90 minutes, and a smaller progressive uptake of potassium by the resin. When the resin, in that state, was transferred from the small to the large intestine, it gradually lost sodium but continued to take up potassium.

Our experiments indicated that, in the dog, progressive restriction of dietary sodium was followed by progressive decreases in the sodium concentrations in the fluid of the terminal ileum. When the restriction of dietary sodium was severe, the concentration of sodium in the terminal ileum was about one-tenth of the normal concentration. As the concentrations of sodium declined, the concentrations of potassium, in the terminal ileum, rose reciprocally, iso-osmolarity being thereby approximately maintained. With restriction of dietary sodium, there was not only less sodium in the terminal ileum with which the resin might combine, but there were also higher concentrations of potassium, for which the resin has a greater affinity than for sodium.[10, 11] The latter placed a further limitation upon the bonds of the resin which were available for combination with sodium.

There was the added effect of hydrogen ions, resulting from bacterial fermination, in the terminal ileum. Reports of such fermentation in the ileum are quoted by Best and Taylor.[14] Hydrogen ions release, by exchange, both sodium and potassium ions from the resin. In the dogs with moderately restricted sodium intake, corresponding to 1,000 mg. per day for an average man, more sodium was free, in solution, than was bound by the resin in the terminal ileum. It is possible that, in the fistula dogs, fermentation in the terminal ileum was augmented by bacteria introduced with the catheter during samplings. The free sodium in the uncontaminated terminal ileum of the sacrificed dog, shown in Table II, was less than the average free sodium of the fistula dogs, while they received the control diet plus resin, but was well within the range of their day-to-day variations.[11]

There are many indications of the active absorption of sodium, against a gradient, in the colon. Most striking, are the reports of Odel and Ferris.[15, 16] They successfully used a solution, in approximate electrolyte equilibrium with the plasma, for dialyzing lavage of the small intestine in the treatment of renal insufficiency. When they used the same solution for dialyzing lavage of the colon, of 40.5 L. of solution run in through an appendicostomy tube in 64 hours, only 24.5 L. were recovered through a rectal tube and their patient became edematous. The colons of our dogs, with fistulas into the terminal ileum, during the administration of resin with diets designed to correspond approximately to 1,000 and 200 mg. of sodium per day, for an average man, absorbed 80 and 88 per cent, respectively, of the sodium that was estimated to have been present in the terminal ileum.[11]

Those deterrents to the retention of sodium in the feces by cation-exchange resin, during restriction of dietary sodium, were fortunate. They operated proportionally to the severity of the sodium restriction. Resins with a greater affinity for sodium, at the pH of the intestinal contents could be used. Without meticulous control, they would be hazardous. In our recently compensated cardiac patients, maintained on Resodec and diets with 800 to 1,200 mg. of sodium per day for 6- or 7-week periods, the per cent of sodium excretion in the feces was 16.0, 30.8, 35.8, 56.8, and 97.5, respectively.[8] The last patient had an average urinary sodium of 13.8 mg. per day. Much of the time it was around 12.5 mg. per day. He maintained a normal serum sodium, but he had a narrow margin of safety.

The mechanism for the widely different percentages of retention of dietary sodium in the feces by those different patients, during cation-exchange resin therapy, was not explored. It was possibly hormonal. It is also possible that our dogs retained a larger proportion of dietary sodium in their feces, during restriction of dietary sodium and resin administration than did our patients on long-term resin therapy, because of the absence of other reasons for alteration of hormone secretion. In 4 of those 5 patients, the amount of sodium retained in the feces was quite significant.

SUMMARY

There are mechanisms which limit, in proportion to the severity of the restriction of dietary sodium, the amount of sodium which is retained in the feces by a cation-exchange resin. They are: (1) Conservation of sodium in the upper gastrointestinal tract with reciprocal increases in the concentrations, in the terminal ileal fluid, of potassium, which has a greater affinity for the resin than has sodium. (2) Bacterial fermentation which provides hydrogen ions to displace both sodium and potassium from the resin in the terminal ileum. (3) Active absorption of sodium, against a gradient, in the colon.

It was fortunate that those mechanisms operated. Otherwise, the use of cation-exchange resins could have produced serious sodium depletion.

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CLINICAL AND LABORATORY OBSERVATIONS ON TWO TRIFLUOROMETHYL PHENOTHIAZINE DERIVATIVES

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ALTHOUGH chlorpromazine (Thorazine) is a potent antiemetic drug, some types of nausea and vomiting do not respond well to this agent and side reactions frequently limit its usefulness. For these reasons further evaluation of antiemetic agents is indicated. The following report presents some observations on the pharmacology and toxicology of two new antiemetic agents, SKF 4648† and SKF 5019,‡ which are more potent than chlorpromazine.[1] These agents are trifluoromethyl derivatives that are related to chlorpromazine.

METHODS AND MATERIALS

Laboratory Observations of Antiemetic Properties.—The antiemetic effects of the experimental preparations, SKF 4648 and SKF 4019, were studied in dogs by giving apomorphine intravenously and copper sulfate orally through a gastric tube. The emetic threshold for apomorphine was determined for each of 10 dogs by giving 5 μg/Kg. intravenously as an initial trial dose. If emesis did not occur, this dose was increased on subsequent days in 5 μg/Kg. increments until vomiting occurred. Although the emetic threshold dose varied from animal to animal, it was always between 15 and 35 μg/Kg.

After the threshold dose of apomorphine was established, each of the experimental antiemetic agents was given on a subsequent day to 5 of the animals, starting with a dose of 0.1 mg./Kg. of body weight. Twenty minutes later the previously established emetic dose of apomorphine was given. The dose of the experimental antiemetic agent was increased 0.05 mg./Kg. on subsequent days until that dose was established at which the emetic response to apomorphine was blocked completely. The results with the two experimental compounds were compared with similar observations made after the same 10 dogs were given 1 mg./Kg. of chlorpromazine.

Ten dogs were also used to observe the ability of each experimental preparation to block intestinal reflex emetic stimuli produced by intragastric copper sulfate. Fifty milligrams per kilogram of copper sulfate produced emesis consistently. Subsequently, the animals were given 1 mg./Kg. of SKF 4648 intravenously. Thirty minutes later a dose of 50 mg./Kg. of copper sulfate was administered and the emetic response to copper sulfate was observed. On a subsequent day similar observations were made, employing a dose of 10 mg./Kg. of SKF 4648. The same procedure was followed in studying the antiemetic effect of SKF 5019.

Laboratory Studies of Renal Hemodynamics and Water and Electrolyte Excretion.—At least one report indicates that water and electrolyte excretion rates are altered by the administration of chlorpromazine.[2] Therefore, it seemed desirable to observe the effect of

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Received for publication May 1, 1957.

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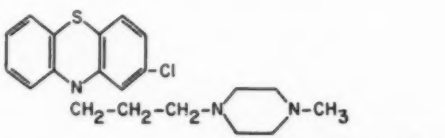
†Available from Smith, Kline & French Laboratories, Philadelphia, Pa.; also available as MC 4703 from E. R. Squibb & Sons, New York, N. Y.

‡Available from Smith, Kline & French Laboratories.

the experimental agents on renal hemodynamics and on water and electrolyte excretion. SKF 5019 was given to 24 unhydrated dogs (Group I), employing doses of 20 mg./Kg. (Subgroup IA), 15 mg./Kg. (Subgroup IB), and 10 mg./Kg. (Subgroup IC). On the morning of the study these dogs were anesthetized with 30 mg./Kg. of pentobarbital and catheterized. Control observations were then made of urine and electrolyte excretion, using three 20-minute urine collection periods. Then the drug was administered intravenously and urine was collected for 4 hours, using 20-minute collection periods as in the control observations.[3, 4] Similar observations were made on 24 unhydrated dogs (Group II), which received SKF 4648 in doses of 5 mg./Kg. (Subgroup IIA), 10 mg./Kg. (Subgroup IIB) and 20 mg./Kg. (Subgroup IIC).

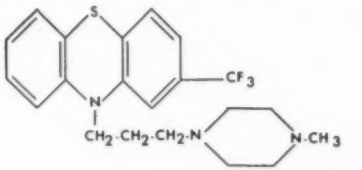
FORMULA OF COMPAZINE



2-Chloro-10-[3-(1-methyl-4-piperazinyl)-propyl]-phenothiazine Dimaleate

Fig. 1.—Structural formula of proclorperazine (compazine).

FORMULA: SKF 5019-A_2



10-(3'-(1''-Methylpiperazinyl-4'')-propyl)
-2-trifluoromethyl-phenothiazine dihydrochloride

Fig. 2.—Structural formula of SKF 5019.

Renal hemodynamics were studied in 16 dogs. Eight (Group III) received 1 mg./Kg. of SKF 5019, and the remaining 8 (Group IV) received 1 mg./Kg. of SKF 4648. The dogs were taken from their cages on the morning of the study and hydrated with 40 ml./Kg. of water. They were then anesthetized with 30 mg./Kg. of pentobarbital. A constant infusion of 5 per cent glucose was given at the rate of 5 c.c./10 Kg. body weight for one hour prior to obtaining the control observations. Glomerular filtration rate was determined by the creatinine clearance and para-aminohippurate clearance was used to determine the renal plasma flow. Mean blood pressure was determined by intra-arterial manometry, methods, and techniques have been described previously.[5] Before giving the experimental compound, we determined the intravenous dose of norepinephrine that would increase the blood pressure 20 to 30 mm. Hg. This same dose of norepinephrine was repeated after giving the experimental drug to detect evidence of adrenergic blockade (reduced response to norepinephrine.

Clinical Observations of Acute Toxicity.—Observations for acute toxicity were made on two groups of patients. Five normal individuals (Group I) received both SKF 5019 and SKF 4648 parenterally. The initial intramuscular dose of each drug was 2.5 mg. On a subsequent day an intramuscular dose of 5 mg. was given. Two or three days after receiving one of the drugs, the subject was given the alternate experimental compound

according to the same dosage schedule. In this way the acute response to SKF 4648 and SKF 5019 was compared in the same patients. Observations of psychic alterations, cardiovascular responses, and effects on the autonomic nervous system were recorded.

The acute response following oral administration was studied in 28 hospitalized patients (Group II). Twelve (Subgroup IIA) received SKF 5019 and 16 (Subgroup IIB) received SKF 4648. Twelve patients were undergoing diagnostic studies for peptic ulcers or were recuperating from pneumonia. Four of these received SKF 5019 and 8 received SKF 4648. Sixteen other patients received the drugs for nausea and vomiting of various etiologies: 8 were given SKF 5019 and 8, SKF 4648. The initial dose of each drug for the control patients without nausea was 5 mg. given orally, followed subsequently by an oral dose of 10 mg. Patients being treated for nausea and vomiting were given 5 mg. intramuscularly every 6 hours. If this dose was inadequate to control nausea and vomiting, a dose of 10 mg. was given by the same route.

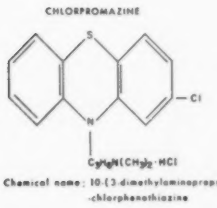


Fig. 3.

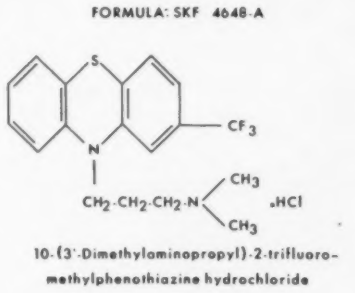


Fig. 4.

Fig. 3.—Structural formula of chlorpromazine (thorazine).
Fig. 4.—Structural formula of SKF 4648.

Clinical Observations of Chronic Toxicity.—

Hospitalized patients (Group III):* In Subgroup IIIA were 13 hospitalized patients (Table II, Group VI) who were being treated for chronic nausea and vomiting, and 4 convalescent patients; all received SKF 4648 for 1 to 4 weeks. Repeated liver function studies included thymol turbidity, alkaline phosphatase, serum bilirubin, and Bromsulphalein excretion. Urinalyses, electrocardiograms, and complete blood counts were done weekly. The daily dosage used to treat nausea and vomiting varied between 10 and 30 mg. The initial dosage given to the 4 convalescent patients was 5 mg. every 6 hours; this dosage was increased gradually to 40 mg. every 6 hours, or until side effects became intolerable. The maximal tolerated dosage was continued for 1 to 2 weeks.

Twelve convalescent patients (Subgroup IIIB) received SKF 5019 in the study of chronic toxicity. The initial dosage, 5 mg. every 6 hours, was increased gradually at 3-day intervals to a maximum of 40 mg. every 6 hours, or until intolerable side effects appeared. The maximal tolerated dosage was continued for 1 to 2 weeks. Studies of liver function, urinary changes, and blood counts were done before the drug was started and at weekly intervals thereafter. These patients were seen by the investigators 4 times daily.

Outclinic patients (Group IV): Twenty-eight patients (Subgroup IVA) who were not vomiting were started on SKF 5019 and 22 (Subgroup IVB) were started on SKF 4648. The initial dosage of each drug was 5 mg. given with each meal and at bedtime. After 5 to 7 days the dosage was increased to 10 mg. 4 times a day and continued for up to 4 weeks. Twenty of the 22 patients in Subgroup IVB received SKF 4648 for 2 to 4 weeks; 15 of these patients received the drug for 4 weeks or more. Twenty-six of the 28 patients in Subgroup IVA received SKF 5019 for 2 to 4 weeks; 20 of these received the drug for 4 weeks

*Data on 12 of these patients were collected by Hugh Bennett, M.D., and Wilson Fraser, M.D., The Veterans Administration Hospital, Houston, Texas.

or more. Control observations were made of Bromsulphalein excretion, alkaline phosphatase, serum bilirubin, thymol turbidity, and cephalin flocculation. After the drug was started, these tests were repeated weekly and at the conclusion of each study. In addition to the liver function studies, complete blood counts, urinalyses, phenolsulfonphthalein excretion tests, and electrocardiograms were done weekly. The patients were examined once or twice a week.

Therapeutic Studies of Patients With Nausea and Vomiting (Groups V and VI).—The antiemetic effects of SKF 4648 and SKF 5019 were observed in the same 16 patients with nausea and vomiting who were used in the acute toxicity studies. Alternate patients were given SKF 5019; the remainder received SKF 4648. After an initial dose of 5 mg., the dose was increased progressively until nausea and vomiting were arrested or side effects appeared. The response was then compared with that obtained with 25 mg. of chlorpromazine. Because SKF 4648 proved to be more effective as an antiemetic than SKF 5019, the antiemetic effectiveness of SKF 4648 was studied in a group of 13 patients with chronic nausea and vomiting (Group VI). These 13 patients were also included in the chronic toxicity studies (Subgroup IIIA), and observations for hematopoietic, cardiovascular, hepatic, and renal toxicity were made in these patients as outlined.

TABLE I. A COMPARISON OF THE ANTIEMETIC EFFECTS OF THE SAME DOSES OF CHLORPROMAZINE, SKF 4648, AND SKF 5019 ON APOMORPHINE-INDUCED EMESIS IN DOGS

	EMETIC RESPONSE TO I.V. DOSE OF APOMORPHINE			
	CHLORPROMAZINE		SKF 4648	SKF 5019
DOG NO.	0.2 MG./KG.	1 MG./KG.	(0.2 MG./KG.)	(0.2 MG./KG.)
1	Vomited	Blocked	----	Retched
2	Vomited	Retched	----	Vomited
3	Vomited	Vomited	----	Blocked
4	Retched	Blocked	----	Blocked
5	Blocked	Blocked	----	Blocked
6	Vomited	Retched	Retched	----
7	Vomited	Blocked	Defecated	----
8	Blocked	Blocked	Blocked	----
9	Retched	Blocked	Blocked	----
10	Vomited	Retched	Blocked	----

RESULTS

Laboratory Observations of Antiemetic Properties.—Laboratory tests of the antiemetic effects of SKF 4648 and SKF 5019 indicated that these drugs were potent inhibitors of apomorphine-induced emesis and were superior to the same doses of chlorpromazine in this respect. Following intravenous administration of 0.2 mg./Kg. of SKF 4648, emesis was inhibited consistently when threshold doses of 15 to 35 μg/Kg. of apomorphine were used. A 0.1 mg./Kg. dose of SKF 4648 was effective in 25 per cent of the dogs and 0.15 mg./Kg. was effective in 75 per cent of the animals. SKF 5019 was not so effective as SKF 4648 in blocking apomorphine emesis, for vomiting was observed occasionally when a dose of 0.2 mg./Kg. was used (Table I). However, at a dose of 0.3 mg./Kg. there was no vomiting and retching was seen only occasionally. At a dose of 0.15 mg./Kg. vomiting was observed in half the dogs. Table I compares the actions of the same doses of SKF 4648, SKF 5019, and chlorpromazine against apomorphine in the same 10 animals on different days. The experimental compounds were ineffective against emesis produced by oral administration of copper sulfate. Doses of 1 mg./Kg. of both SKF 4648 and SKF 5019 had no observable antiemetic effect against 50 mg./Kg. of intragastric copper sulfate. Following doses of 10 mg./Kg. of the experimental drugs the animals presented

a characteristic response, which was more marked with SKF 5019 than with SKF 4648. First, the animals stood with their feet spread apart and their heads down. They were unresponsive to stimuli and showed no reaction to the investigators. Later they lost their balance and appeared to be inebriated. Even at this dose (10 mg./Kg.) neither drug was very effective against nausea and vomiting produced by intragastric copper sulfate. The severity of the vomiting was reduced, but all the dogs salivated profusely and 9 of the dogs that received SKF 5019 vomited; 8 of the 10 that received SKF 4648 vomited.

*Laboratory Observations of Renal Hemodynamics and Water and Electrolyte Excretion.**—Following the administration of doses of 10 to 20 mg./Kg. of SKF 5019 to 24 unhydrated dogs (Group I) there was no consistent effect on urine volume or sodium and potassium excretion. Occasionally during the follow-up period there were variations from the control values that approached statistical significance. But these were extremely erratic and probably were fortuitous variations. There was no consistent effect on water or electrolyte excretion rates after the administration of SKF 4648 (Group II). As with SKF 5019, there was some slight, but inconsistent, variation from control values in sodium and potassium excretion. We can conclude that the trifluoromethyl derivatives do not appear to affect water and electrolyte excretion significantly or consistently within 3 hours after intravenous administration.

Following the administration of 1 mg./Kg. of SKF 5019 (Group III) there was no significant effect on blood pressure and no effect on glomerular filtration rate. After 2 hours renal blood flow was slightly depressed, but the decrease was no greater than one would see after 2 hours of deep anesthesia without concurrent drug administration. Certainly there was no significant or immediate effect on renal hemodynamics. The administration of both SKF 5019 and SKF 4648 always deepened the anesthesia. Following the administration of SKF 4648 (Group IV) there was a temporary reduction in blood pressure from 102 to 84 mm. Hg ($p < 0.01$), but by the end of one hour the blood pressure began to increase, and at the end of two hours it had returned to control levels. We conclude that apparently SKF 4648 is more likely to reduce blood pressure than SKF 5019.

Adrenergic blockade was minimal following the administration of SKF 5019 in a dose of 1 mg./Kg., but was more evident following the administration of 1 mg./Kg. of SKF 4648. In no animal was there evidence of epinephrine reversal or complete norepinephrine blockade. In fact the response to norepinephrine was always 50 per cent or more of that observed prior to the administration of SKF 4648 and SKF 5019, using the same dose of norepinephrine.

Alterations of urine volume and sodium excretion were not significant following the administration of SKF 5019 to the hydrated dogs. However, there did seem to be a slight increase in potassium excretion from 23 μEq to 39 μEq/min. ($p < 0.01$). This response was inconstant and of no great magnitude.

*Material supplementary to this article has been deposited as document number 5374 with the ADI Auxiliary Publications Project, Photoduplication Service, Library of Congress, Washington 25, D. C. A copy may be secured by citing the document number and by remitting $1.25 for photoprints or $1.25 for 35 mm. microfilm. Advance payment is required. Make checks or money orders payable to: Chief, Photoduplication Service, Library of Congress.

Following the administration of SKF 4648 there was an increase in urine volume. This was significant ($p < 0.01$) throughout the experiment although the magnitude was not great. A temporary increase in sodium excretion disappeared within the first hour after drug administration. There were no significant effects on potassium excretion or on the plasma concentration of sodium.

Clinical Observations of Acute Toxicity.—

Intramuscular administration (Group I): After intramuscular administration the onset of drug action appeared within 30 minutes and reached a plateau after approximately one hour. The only side effects noted in these 5 normal subjects following the administration of 2.5 mg. of SKF 4648 were mild sedation and somnolence in 2 patients. However, the same dose of 2.5 mg. of SKF 5019 given intramuscularly to the same patients caused all but one to become drowsy, and in one instance the somnolence was marked. It was quite evident that the soporific effect of SKF 5019 was considerably greater than that of SKF 4648. In none of the patients was there any hemodynamic effect in either the supine or the upright position.

When a dose of 5 mg. of either drug was employed, patients complained of somnolence, weakness, "arms of lead," and inability to think clearly. Two patients receiving SKF 5019 became restless and apprehensive. One of these became agitated and said that he "felt wild inside." When given a placebo injection the next day, he did not obtain such a response. Three patients receiving SKF 5019 and one patient receiving SKF 4648 complained of marked dizziness with no observable effect on blood pressure. These side effects were more marked with SKF 5019. There was a significant orthostatic reduction in blood pressure (not observed in the supine position) in one patient after receiving SKF 4648 but the blood pressure was not affected in any patients receiving SKF 5019.

Oral administration (Group II): Results with SKF 5019: Of the 12 patients in Subgroup IIA, one developed tachycardia and marked sweating within one hour after taking 5 mg. of the drug. In another the blood pressure dropped from 120/80 in the standing position to 80/60 after the administration of 10 mg. Hypotension was not observed in the supine position. The psychic effects of SKF 5019 were quite prominent. Five patients complained of being nervous and apprehensive. Two noted dizziness and one, blurred vision. One complained of a thick tongue and was unable to speak properly. Seven of the 12 patients felt sleepy, but 6 could only sleep for short periods, which they described as "cat naps." When they awoke, they did so with a start. These effects usually cleared after 4 to 6 hours, but two patients complained of nervousness and apprehension for 12 hours. The patients looked pale and expressionless, and their emotional response was neither normal nor appropriate. They appeared very restless, and yet their actions were slow and frequently seemed to lack purpose. There was no great difference between the effects of the 5 and the 10 mg. doses.

Results with SKF 4648: Of the 16 patients in Subgroup IIB, two developed a temporary increase in pulse rate (from 80 to 100 in one and from 72 to 96 in the other). Hypotension was observed in 3 patients but occurred only in

the standing position in two instances. There were no electrocardiographic changes other than sinus tachycardia. Five patients became quite drowsy within one hour after taking the drug. Two patients complained of extreme weakness and two of dizziness although the blood pressure was not reduced. Dry mouth was noted by one patient. Two patients complained of a "panic" sensation. They felt very restless, and one said that she "felt as though (she were) someone else"—a sense of losing contact with her environment. Another patient had blurred vision. The psychic responses in general were neither so frequent nor so severe as they were in the group of patients receiving similar dosages of SKF 5019. The incidence of side effects was the same after taking 5 mg. as after taking 10 mg., but the severity of the response was slightly greater after the larger dose.

Clinical Observations of Chronic Toxicity.—

Hospitalized patients (Group III): The 13 hospitalized patients in Subgroup IIIA with chronic nausea and vomiting received 10 to 30 mg. of SKF 4648 (Table II for 1 to 3 weeks). There was no evidence of renal or cardiovascular toxicity except for tachycardia in one patient. In 2 patients the thymol turbidity was elevated to 8 units but remained elevated for only one week, despite continued drug administration. There was no clinical evidence of hepatic toxicity. Central nervous system effects, somnolence, weakness, dry mouth, and dizziness, decreased as therapy was continued. However, 2 patients complained of irritability, nervousness, and insomnia which did not improve with continued medication. If anything, these effects increased in severity.

The 4 convalescent subjects in Subgroup IIIA who received SKF 4648 in increasing dosages up to 40 mg. 4 times a day showed no greater incidence of side effects at the maximum dosage than at dosages of 10 to 20 mg. given 4 times a day. The only side effects noted in these 4 patients were drowsiness and fatigue in 2 and orthostatic hypotension in another. These effects disappeared after 2 days of drug administration in spite of the increasing dosages.

CASE 1.—A 54-year-old woman who was being studied for allergic rhinitis was given 5 mg. of SKF 5019 4 times daily. At the beginning of therapy the blood pressure was 170/75 in both the supine and standing positions. After one week she said that SKF 5019 was almost intolerable and complained of mild dizziness, moderate sedation, and a marked fatigue-like state. She also stated that after 3 days of SKF 5019 therapy she was unable to chew; her tongue had become heavy and difficult to coordinate. She noted slight nuchal rigidity and developed considerable numbness of both arms. She stated that she was very nervous and was unable to sleep at night. She was given an identical placebo and one week later the blood pressures were recorded as 174/74 (supine) and 176/100 (standing). She showed symptomatic improvement and felt much stronger.

The 12 convalescent patients in Subgroup IIIB were started on dosages of 5 mg. of SKF 5019 every 6 hours, but 4 refused medication after two doses because of nausea (1 patient), nervousness and blurred vision (2 patients), and nervousness and insomnia (1 patient).

The remaining 8 hospitalized patients received SKF 5019 in dosages up to 20 to 40 mg. 4 times daily. The maximal dosage was continued for one to two weeks. In 4 of these patients the dosage was increased only to 20 mg. 4 times a

TABLE II. THERAPEUTIC RESPONSE TO SKF 4648 IN PATIENTS WITH NAUSEA AND VOMITING OF VARIOUS ETIOLOGIES

PATIENT	AGE	SOURCE OF EMESIS	SEVERITY	DOSE (MG.)	SKF 4648 DURATION OF THERAPY (DAYS)	THERAPEUTIC ANTI-EMETIC RESPONSE	CHANGES IN BLOOD PRESSURE OR HEART RATE	HEMATOPOIETIC CHANGES	ALTERATIONS IN LIVER FUNCTION	SIDE EFFECTS
1. M. B.*	24	Tetracycline—uremia and pyelonephritis	Moderate	20	13	Good	None	None	Thymol 8; others normal	Drowsiness, weakness
2. M. B.†	24	Uremia and pyelonephritis	Severe	30‡	7	Poor	None	None	None	None
3. P. G.	47	Amebic abscess treated with emetine, chloroquine, and tetracycline	Severe	20§	7	Excellent	None	None	None	Drowsiness, dry mouth, skin rash
4. M. N.	57	Carcinomatosis	Moderate	15§	14	Excellent	None	None	None	None
5. W. F.	35	Uremia and renal tuberculosis	Severe	10‖	23	Excellent	None	None	Thymol 8; others normal	None
6. Y. M.	15	Psychogenic	Moderate	20	14	Excellent	None	None	None	None
7. A. P.	14	Infectious	Moderate	20	5	Excellent	None	None	None	None
8. J. C.	46	Alcoholic gastritis, portal cirrhosis	Moderate	20§	27	Excellent	None	None	None	Blurred vision, drowsiness, dizziness
9. F. S.	48	Cirrhosis, postoperative	Severe	20	7	Excellent	None	None	None	Drowsiness, insomnia, detached, restless, weak
10. C. B.	32	Disseminated lupus erythematosus	Severe	30§	14	Excellent	Increased heart rate	None	None	Somnolence
11. A. P.	36	Disseminated lupus erythematosus	Severe	20§	12	Good	None	None	None	Severe insomnia, irritability, nervous detachment
12. M. M.	31	Pregnancy	Moderate	20	21	Good	None	None	None	Somnolence, detatched
13. M. F.	30	Pregnancy	Moderate	10	7	Fair	None	None	None	Nervous, panic feeling
14. K. K.	24	Pregnancy	Mild	10	7	Excellent	None	None	None	None

Therapeutic Response Rating: Excellent = complete arrest of nausea and vomiting; Good = arrest of vomiting with intermittent nausea; Fair = vomiting arrested but nausea continues; Poor = no therapeutic response.

*Same as Subject 2, first admission.

†Second admission.

‡Intramuscular administration.

§Previously treated with 25 mg. chlorpromazine every 4 hours with only fair or poor results.

‖Initially administered intramuscularly, subsequently increased to 15 mg. daily by mouth.

day; these patients refused larger amounts because of the nervousness, apprehension, and anxiety they developed with larger dosages. All the symptoms subsided when placebos were substituted for the experimental drug. Of the remaining 4 patients who received dosages of 40 mg. 4 times a day, 2 complained of nervousness, sweating, and apprehension. The side effects in each patient disappeared on 2 different occasions when placebos were substituted. The remaining patients experienced no symptoms attributable to the drug. There was no evidence of renal, hematopoietic, or hepatic toxicity in any of these patients.

TABLE III. SIDE EFFECTS WITH LONG-TERM USE OF SKF 5019 AND SKF 4648 IN OUTPATIENTS

	GROUP IVA SKF 5019		GROUP IVB SKF 4648	
	(NO.)	(PER CENT)	(NO.)	(PER CENT)
Number of patients treated	28		22	
Drowsiness	18	64	8	36
Agitation	8	29	0	—
Weakness	10	36	2	9
Dizziness	10	36	2	9
Dry mouth	4	14	2	9
Salivation	2	7	—	—
Blurred vision	4	14	0	—
Parkinsonism	3	11	1	5
Insomnia	9	32	5	23
Spasm of jaw muscles	2	7	1	5
Thick tongue	1	4	—	—
Torsion spasm (torticollis)	3	11	1	5
Leg cramps	2	7	1	5

Outclinic patients (Groups IV): A summary of the side effects appears in Table III and the laboratory and cardiovascular effects are shown in Table IV. About one-third of the patients receiving SKF 4648 and two-thirds of those receiving SKF 5019 complained of drowsiness. About 10 per cent of those receiving SKF 4648 and one-third of those receiving SKF 5019 complained of weakness, dizziness, restlessness or agitation, and an inability to sleep in spite of somnolence and fatigue. Drowsiness became so marked in 4 patients receiving 40 mg. per day of SKF 5019 that the drug had to be discontinued.

CASE 2.—A 38-year-old white woman came to the clinic because of chronic anxiety. She was given SKF 5019 and told to take 5 mg. with each meal and at bedtime. Prior to administration of the drug her pulse rate was 84; blood pressures were recorded as 170/100 (supine) and 140/90 (standing). After she took the drug for one week, her anxiety decreased; blood pressures were 158/102 (supine) and 140/90 (standing). The sedative response she observed seemed to be about the same as that with a 30 mg. dosage of phenobarbital 4 times a day. The dosage of SKF 5019 was increased to 10 mg. 4 times a day, but the patient discontinued the drug on the third day because of side effects. She became markedly lethargic, was unable to sleep at night, and noticed marked dryness of the mouth. Her insomnia was associated with agitation and inability to relax. She frequently fell asleep during the day, but within several minutes she awoke with a start. Laboratory observations, including blood and liver function studies, showed no abnormalities.

Most of the side effects that persisted for 4 weeks or more with administration of SKF 4648 or SKF 5019 did not appear when the patient was switched unknowingly to a placebo. Therefore, it seems evident that these untoward

effects were attributable to the drugs. In general the side effects initially present tended to diminish with continued drug administration. All patients were given an initial daily dosage of 20 mg. (5 mg. 4 times daily) of SKF 5019 or SKF 4648. While increasing the dosage to 40 mg. daily did not increase the incidence of side effects, their severity frequently increased.

TABLE IV. SUMMARY OF LABORATORY OBSERVATIONS AND CARDIOVASCULAR RESPONSE IN PATIENTS ADMINISTERED SKF 4648 AND SKF 5019 FOR 2 TO 4 WEEKS
(Expressed as number of patients receiving drug)

	GROUP IVA (SKF 5019)		GROUP IVB (SKF 4648)	
	PERIOD D_1*	PERIOD D_2†	PERIOD D_1*	PERIOD D_2†
Number of patients studied	26	20	20	15
Hypotension‡	0	0	2	0
Hypertension§	2	1	0	0
Tachycardia‖	2	0	2	0
Hematological changes¶	0	0	0	0
Urinary changes**	0	0	0	0
Liver function studies††	6‡‡	2‡‡	2§§	1‖‖

*Observations during first two weeks of drug administration.

†Observations during and following second 2 weeks of drug administration.

‡Fall in diastolic blood pressure of 10 mm. Hg (or more) after one week of at least 20 mg. daily of SKF 5019 or SKF 4648.

§Increase in diastolic blood pressure of 10 mm. Hg (or more) after one week of at least 20 mg. daily of SKF 5019 or SKF 4648.

‖Increase in pulse rate of 10 beats/min. (or more) after one week of at least 20 mg. daily of SKF 5019 or SKF 4648.

¶Includes R.B.C., hematocrit, W.B.C., and differential.

**Includes acidity, specific gravity, protein, sugar, acetone, and microscopic examination.

††Includes bilirubin, thymol turbidity, alkaline phosphatase, total serum protein (and A/G ratio), and Bromsulphalein excretion.

‡‡Thymol turbidity ranging from 7 to 15 units; other tests within normal limits. No clinical evidence of hepatic derangement.

§§Thymol turbidity of 11 units and other studies normal after 2 days' treatment.

‖‖Thymol turbidity of 7 units.

Hepatic function studies, including thymol turbidity, serum bilirubin, and Bromsulphalein excretion tests, were done on these patients before administering the drugs and at weekly intervals during therapy. Six patients who received SKF 5019, and 2 who received SKF 4648, for 2 weeks or more showed abnormal thymol turbidity tests. The abnormality was of low order: no more than 15 units in any patient. By the end of 4 weeks the thymol turbidity was within normal limits in all but 2 patients receiving SKF 5019 and in all but one receiving SKF 4648, even with continued drug administration. There was no other evidence of hepatic derangement, and in every case the thymol turbidity returned to normal after the drug was stopped. It is possible that the drug interferes chemically with the thymol turbidity test; this possibility was not investigated. Urinalyses repeated at weekly intervals showed no evidence of renal toxicity (Table IV). Complete weekly blood counts on these patients throughout the study showed no evidence of blood dyerasias. Electrocardiographic studies failed to show alterations from normal.

The effectiveness of these drugs for the treatment of anxiety and tension in 16 patients is recorded in Table V, which reflects the examiners' interpretation of the subjective response. The patients were selected from the chronic toxicity group.

TABLE V. SYMPTOMATIC RESPONSE IN PATIENTS WITH ANXIETY AND TENSION

RESPONSE	GROUP IVA SKF 5019		GROUP IVB SKF 4648	
	(NO.)	(PER CENT)	(NO.)	(PER CENT)
Number of patients treated	11		5	
Good	5	45	2	40
Fair	1	9	1	20
Poor	5	45	2	40

The number of poor responses following administration of these agents is probably a result of the high rate of agitation observed with the use of these drugs. All but two patients were given either a placebo or phenobarbital in identical capsules. Thus, while clarification of good, fair, and poor is vague, the classification serves as a comparison with placebos and phenobarbital.

That these drugs potentiate barbiturates is indicated by the following case history:

CASE 3.—A 55-year-old white man with mild anxiety and tension was given a dosage of 2 mg. of SKF 5019 4 times daily. This patient also took 200 mg. of sodium amytal, which he had used for years for insomnia, apparently with no previous difficulty. However, when he took sodium amytal concurrently with the SKF 5019, he developed such deep somnolence that he could not be aroused for 18 hours. He remained markedly lethargic and dizzy for an additional 48 hours and was unable to walk by himself to the bathroom. Subsequently he recovered and after 4 days felt completely normal.

Therapeutic Studies of Patients With Nausea and Vomiting (Groups V and VI).—When their antiemetic effects were compared (Group V), SKF 4648 was found to be superior to SKF 5019. Doses of 5 mg. of SKF 4648 appeared to be adequate for arresting nausea and vomiting in most cases. In contrast to results with chlorpromazine, the antiemetic effect persisted for 8 to 10 hours in some instances. When SKF 4648 was given every 6 hours, the second dose was usually more effective than the first. SKF 5019 appeared to be about equally as effective as chlorpromazine but psychic side effects were more marked.

The results obtained with SKF 4648 in the treatment of chronic nausea and vomiting (Group VI) are summarized in Table II. The drug failed to arrest nausea and vomiting in only one instance. A uremic patient (M. B.) had responded to the drug and, in fact, had been discharged from the hospital. Readmitted two weeks later with severe vomiting, she no longer responded to SKF 4648, nor were SKF 5019 or chlorpromazine effective.

Side effects associated with the long-term use of SKF 4648 for the treatment of nausea and vomiting consisted of drowsiness, dizziness, weakness, and blurred vision. These effects tended to increase in intensity during the first day or two of drug administration, apparently the result of a cumulative effect. However, after one week the intensity of the side effects had decreased, although a sense of detachment seemed to persist and frequently was more apparent to the examiner than to the patient.

CASE 4.—A 58-year-old Mexican man with cirrhosis, portal hypertension, and esophageal varices had hemoptysis on numerous occasions. Three days following ligation of the esophageal varices an attempt was made to get the patient to resume a controlled diet. He was

unable to tolerate milk, antacids, or soft foods and nausea and vomiting persisted. Therefore, he was given 5 mg. of SKF 4648 intramuscularly. After 3 hours the nausea and retching were completely arrested. He continued to receive 5 mg. every 6 hours for 4 days without recurrence of the nausea and vomiting. He was able to tolerate solid and semisolid foods, antacids, or a combination of equal parts of milk and cream. Because supplies of SKF 4648 became exhausted, the drug was discontinued. Thirty-six hours after the last dose the patient became nauseated and retched severely. He developed acute left upper quadrant pain and hypotension. The patient was then placed on 5 mg. of SKF 5019, which arrested the nausea and vomiting. He continued to receive 5 mg. every 6 hours for 5 days, and there was no recurrence of nausea and vomiting.

CASE 5.—A 35-year-old Negro man with active pulmonary and renal tuberculosis developed progressive uremia and nausea and vomiting. The patient was treated with a cation exchange resin, which produced a pernicious vomiting that failed to respond to SKF 4648. He was given an intramuscular dose of 5 mg. of SKF 4648 twice daily. There was an immediate and dramatic effect, with excellent improvement of nausea and vomiting. Since then he has been able to retain the cation exchange resin. The blood urea nitrogen was 128. He was switched to SKF 4648, administered orally twice daily, and had an excellent therapeutic response without alterations of the blood pressure or pulse rate. The cation exchange resin was continued for two weeks. The blood urea nitrogen decreased, but he developed progressive nausea without vomiting. Because the patient was evidently developing a tolerance to the drug, the dosage was increased to 5 mg. 3 times daily. Again, the nausea was completely relieved without significant hemodynamic changes. This patient continued to receive a dosage of 5 mg. 3 times daily for 3 weeks, and the blood urea nitrogen returned to normal. When the drug was discontinued, there was no further nausea and vomiting. It is probable that in this case SKF 4648 was a lifesaving therapeutic agent.

CASE 6.—A 47-year-old Latin American man with an amebic abscess of the liver had nausea and vomiting and was unable to retain nourishment or medication. Having been treated with emetine, chloroquine, and Diodoquin, he was given chlorpromazine, 25 mg. 3 times daily, which provided fair control of emesis. Because a skin rash developed with considerable drowsiness and moderate dizziness, the chlorpromazine was discontinued. Nausea and vomiting returned, and the patient was unable to retain oral medication. Therefore, he was given a dosage of 5 mg. of SKF 4648 four times daily. At no time after the initiation of SKF 4648 did he vomit, although he remained nauseated for about 36 hours. Treatment with SKF 4648 was continued for one week, but was discontinued when he developed dryness of the mouth and a peculiar inability to chew or to open his mouth. The attending physician thought this to be an untoward effect of the drug and resumed treatment with chlorpromazine, 25 mg. 3 times daily. This time chlorpromazine provided adequate control of the nausea and vomiting.

COMMENTS

It appears that substitution of the trifluoromethyl component for the chlorine radical greatly enhances the potency of these compounds (Figs. 1 to 4). SKF 4648 has the same structure as chlorpromazine except that the CF_3 group has replaced the chloride in the phenothiazine nucleus. Our observations indicate that SKF 4648 is at least 5 times more potent an antiemetic agent than chlorpromazine. In laboratory animals and in humans it causes no renal or cardiac toxicity. There is no evidence of altered renal hemodynamics or of variation in water and electrolyte excretion following administration of the drug to anesthetized dogs. These observations agree with previous results obtained with chlorpromazine in unanesthetized patients.[2, 6]

The psychic responses to these drugs are the most disturbing side effects, particularly the agitation, anxiety, and insomnia, which frequently occur at night. These effects may be desirable when the drugs are used in the treatment of specific psychiatric problems but have considerable nuisance value when they occur in patients with nausea and vomiting who have no psychiatric complaints. These side effects increase in intensity when therapy is continued for several days, but when drug administration is continued beyond this point tolerance apparently develops and the intensity of the side effects decreases.

It appears that adrenergic blockade is less prominent following the administration of SKF 4648, and especially following SKF 5019, than after chlorpromazine. Therefore, hypotension will probably be observed less frequently.

SUMMARY AND CONCLUSIONS

1. Two trifluoromethyl phenothiazine derivatives (SKF 4648 and SKF 5019), related to chlorpromazine and proclorperazine, were studied in the laboratory and in the clinic for evidence of toxicity and for antiemetic effectiveness.

2. SKF 4648 appears to be more potent as an antiemetic agent than chlorpromazine and SKF 5019.

3. There was no evidence of renal or cardiovascular toxicity following the administration of SKF 4648 and SKF 5019. When given intravenously to unhydrated dogs, these agents did not affect water and electrolyte excretion significantly.

4. There was no evidence of hepatic toxicity except for an occasional slight increase in thymol turbidity. This increase was always minor and was not persistent. Its significance is unknown, but possibly the experimental preparations interfere with the test by a direct or indirect effect on fat metabolism.

5. Psychiatric side effects are the most annoying side effects when these drugs are used as antiemetic agents. However, it may well be that these so-called side effects may become valuable assets when the agents are used to treat psychiatric illness.

6. SKF 4648 is a potent antiemetic, which is more effective than chlorpromazine. However, because of the side effects it should be reserved for patients with severe nausea and vomiting who do not respond to antiemetic agents which have less severe side effects.

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THE FUNCTION AND CAPACITY OF THE ADRENAL CORTEX IMMEDIATELY BEFORE RADIATION SICKNESS DEATH

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INTRODUCTION

DURING the past decade, both the medical literature and textbooks have referred to a variety of clinical conditions following irradiation, infections, intoxications, burns, and other noxious agents as being associated with adrenocortical insufficiency, although these conditions do not present the classic picture of Addison's disease. One also encounters the equivocal phrase "adrenal exhaustion,"[1] employed in many diseased states to suggest that the cortex is the critical organ and that its failure is responsible for the death of the organism. Usually, the evidence for insufficiency or exhaustion is based upon symptomatology, metabolic disturbances, and such other indirect indices of adrenocortical function as hemograms and adrenal histology, ascorbic acid, and cholesterol levels. While most of the alterations, and particularly the adrenal histologic and histochemical ones, are undoubtedly indicative of an active involvement of the pituitary-adrenal axis, they are either unreliable or inadequate as criteria for assessing the actual functional status of the cortex. Aside from a depletion in functional capacity, the development of an adrenocortical dysgenesis[2] has also been proposed in many terminal pathologic conditions in which there is little or no evidence that an abnormal corticosteroidogenesis occurs.

The foregoing hypotheses of exhaustion and dysgenesis prompted the author to study the function and capacity of the adrenal cortex at the time of death from a fatal stressor. The experimental design involved exposure of the calf to a lethal dose of whole-body gamma radiation, removal of both adrenals immediately before radiation sickness death, and perfusion of the glands with ACTH in order to ascertain the nature and quantity of their corticosteroidal output in response to maximal corticotropic stimulation. By removing the adrenals from the intact animal and subjecting them to an in vitro functional test, an opportunity was afforded to investigate directly the adrenocortical steroidogenic mechanism and products without such possible complicating in vivo factors as a fluctuating adrenocortical activating mechanism and/or an altered or deranged corticoid metabolism of damaged, nonadrenal tissues. The markedly enlarged adrenals of the moribund calves were compared not only to control glands but also to adrenals of comparable size obtained from calves of similar age and weight which had been pretreated with ACTH.

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The opinions or assertions contained herein are the private ones of the writer and are not to be construed as official or reflecting the views of the Navy Department or the Naval Service at large.

Received for publication June 19, 1957.

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As a result of the similarity of the terminal symptoms and metabolic disturbances of radiation sickness to those of Addison's disease, it has frequently been alleged that adrenocortical insufficiency contributes to the development of radiation sickness,[3, 4] and that "adrenal exhaustion" may occur terminally.[5] While it was of interest to evaluate this hypothesis, exposure to a lethal dose of ionizing radiation was selected as the stressful stimulus in the present studies mainly because it eventuates in a complex of profound pathologic sequelae, e.g., infection, hemorrhage, anemia, toxemia, and electrolyte, water, acid-base, and other metabolic disturbances, each of which constitutes a severe stress and together produce the radiation sickness syndrome. Then, it was felt that the findings would not only be pertinent to radiation as a noxious agent but also would provide some information concerning the magnitude and nature of the terminal changes in adrenocortical function and capacity which may occur subsequent to other fatal types of stresses.

MATERIALS AND METHODS

Animals.—The 26 male, Holstein-Friesien calves represented in these studies were obtained from a local farm in groups of from 7 to 9 animals, fairly uniform in age and weight and in apparent good health. Prior to delivery, all were immunized with hemorrhagic septicemia bacterin and maintained for at least a week on a commercial feed and roughage. Upon arrival, they were examined, labeled and weighed, and housed thereafter in a dry, roomy shed, which was bedded daily with straw and temperature-regulated so as not to fall below 58° F. All of the present studies were conducted during the winter period from the end of October through February. The animals were allowed fresh water and a good-quality, timothy-clover hay mixture ad libitum, and each was fed twice daily with a quart of commercial feed (Purina Calf Startena). During the observation and acclimatization period of at least two weeks in each experiment, all the calves which were used had gained weight and were free from disease as determined by physical examination and normal hemograms and body temperature.

Irradiation.—On the morning of irradiation, the calves were weighed and each experimental animal was carefully paired as to both age and weight with a control. These pairs were immobilized by hog-tying rather than anesthesia in order to preclude possible complications attendant upon the use of an anesthetic agent. The calf to be exposed was placed in an upright position within a plywood cradle. The cradle was then centered within the cylindrical irradiation chamber of the radiocobalt large animal irradiator[6] at the Naval Medical Research Institute, and the calf exposed to 600 r gamma radiation from cobalt^{60} in a 4 π exposure field. During the course of the investigation, the dose rate ranged from 44.3 to 41.0 roentgens per minute in air, and the exposure time from 13.5 to 14.6 minutes. At the end of each irradiation, the exposed animal and its paired control were promptly untied. It should be added that they remained calm while trussed for a total period of about 25 minutes, and appeared unaffected as evidenced, in part, by their immediate response to feeding.

ACTH Treatment.—Two or three calves, maintained concurrently with the irradiated animals and their untreated controls, were injected intra-muscularly each morning for 8 days with 150 I.U./100 Kg. of ACTH. The first and last doses administered were Acthar,* dissolved in approximately 10 ml. of 0.9 per cent NaCl, and the 6 others were purified corticotrophin in 20 per cent gelatin.† It was hoped that this arbitrarily selected dosage schedule would induce in the ACTH-treated calves an adrenal hypertrophy comparable to that observed terminally in the fatally irradiated animals.

Observations and Procedures.—Body weight was determined upon arrival of the animals, at zero day, and at the time of sacrifice or spontaneous death. During the postexposure and

*The Armour Laboratories, Kankakee, Ill.
†National Drug Company, Philadelphia, Pa.

ACTH-treatment periods, the animals were observed several times daily. Venous samples of free-flowing jugular blood were routinely drawn in the morning and blood counts done at least three times during the surveillance period and at variable intervals thereafter. The hematologic determinations included total white blood cell, differential, direct eosinophil, and platelet counts as well as the volume of packed red cells (VPRC). Complete autopsies were performed on most of the animals, and the gross pathologic observations were supplemented by histopathologic studies of various organs.

Adrenal Perfusion.—The adrenals were excised from the ACTH-treated animals either 24 or 48 hours after the last dose, and from the control and irradiated animals when the latter had either died spontaneously or were moribund. The terms moribund, dying, terminal, agonal, or in extremis have been used synonymously in this report to represent an irreversible condition which would terminate in death within 6 hours or less. The diagnosis of the condition of the exposed calves as moribund or terminal was obviously subjective and based on the clinical experience and impressions derived from previous studies with 23 fatally-irradiated calves maintained until spontaneous death. The animal to be sacrificed was felled by a bullet to the brain and then hoisted by the hind legs to a vertical position. After the jugular vein was severed and the abdomen opened, both adrenals were excised, quickly trimmed, and placed immediately in an ice-cold citrate-saline solution. The total time which elapsed between death and removal of the glands was never more than 15 minutes either in the sacrificed animals or those dying spontaneously.

The two cooled glands of each calf were dissected free of periadrenal fat and connective tissue, weighed, and cannulated via the adrenal vein. They were then perfused in parallel for two hours in a multicycle system at 37.5° C. The perfusion fluid was a liter of an artificial medium, supplemented with 10 I.U./hr. of ACTH* and composed of the following substances, in grams per liter: glucose 2.00, NaCl 8.20, KCl 0.25, $CaCl_2$ 0.20, $MgSO_4$ 0.13, $NaHCO_3$ 0.60, NaH_2PO_4 0.05. It was treated with a continuous stream of water-washed 95 per cent O_2/5 per cent CO_2 and was supplemented with penicillin G (100,000 units/L.) and streptomycin (0.1 Gm./L.) to ensure bacteriostasis. The details of the perfusion technique have been presented elsewhere.[7] At the termination of each perfusion, the collected adrenal effluents were treated with a stream of nitrogen and immediately frozen in a Dry Ice-acetone mixture. They were kept in this state until extracted for corticoids.

Perfusate Extraction and Analysis.—Each thawed perfusate was extracted serially with four 300 ml. portions of cold ethyl acetate. The combined extracts were washed with 50 ml. of water, 50 ml. of 0.1 N NaOH, and finally with three 50 ml. portions of water. The extract was then dried with sodium sulfate and evaporated under vacuum to a residue. This steroidal residue was then fractionated by paper partition chromatography, and the eluates of the various zones assayed by the Porter and Silber[8] (cortisol and cortisone) and phosphomolybdic acid (corticosterone) reactions.[9] These assays were confirmed by reaction with alkaline blue tetrazolium.[10] The extraction, chromatography, and assay procedures employed have already been described in detail.[11, 12]

Adrenal Water and Nitrogen.—The adrenal moisture content was calculated from the initial wet weight and the weight obtained after drying in vacuo over P_2O_5 for 2 days and at 80° C. for 24 hours. The dried adrenal tissue was pulverized to a fine powder and the latter passed through three layers of a fine-meshed screen to remove the flakes of capsular tissue. The nitrogen content of duplicate weighed samples of these adrenal powders was determined by the micro-Kjeldahl method, as modified by Eisner and Wagner.[13]

RESULTS

Clinical, Mortality, and Pathology Data of Irradiated Animals.—The data of a preliminary report[14] indicated that the whole-body dose of 600 r represents the $LD_{40/9}$ or the $LD_{100/14}$ for male calves of a weight and age comparable to those presently employed. The postirradiation clinical course, body weight,

*Acthar, The Armour Laboratories, Kankakee, Ill.

temperature, and hematologic changes as well as gross and microscopic pathology were also presented in detail in this report.[14] Regarding the exposed animals of the present studies, it is pertinent to note that, after an asymptomatic phase lasting until the fifth or sixth postirradiation day, they exhibited diarrhea and anorexia of varying intensity and persistence. Weight loss, hyperpyrexia, profuse weeping, a wobbly gait, pronounced apathy and malaise, and marked respiratory distress with excessive salivation were the most prominent symptoms observed terminally in all the animals, and were manifested in about half of them after the ninth day.

Upon autopsy, all the calves which had died spontaneously or were killed in a moribund condition exhibited petechiae and hemorrhagic streaks throughout the viscera and parietes, and areas of hemorrhagic consolidation in the lungs. Their red bone marrow appeared drier and paler than normal, and the lymph nodes, essentially devoid of lymphocytes, were more black than gray. In addition to hemorrhage, the lungs manifested congestion, edema, and bacterial colonies indicative of infection. Bacterial implantation was also observed in the liver and in the lesions of the spleen, lymph nodes, thymus, and Peyer's patches. Regarding the terminal phase of hemorrhage and overwhelming sepsis, it is significant to note that the platelet count declined linearly after the fifth day, approaching zero around the eleventh day after exposure; the leukocyte count, after a mean initial rise from a pre-exposure level of 8,000/mm.^{3} to 14,800/mm.^{3} in 12 hours, dropped precipitously to 1,600/mm.^{3} by the second day. Thereafter, the number of circulating leukocytes declined consistently until exitus with no tendency for a rebound. The decrease in the hematocrit values, which were found to be below normal in most of the irradiated animals by the ninth day, was related mainly to the severity of the generalized hemorrhagic condition. In general, the sequence of the pathologic changes and the microscopic lesions observed were similar to those described in other mammals following a lethal dose of ionizing radiation.

Clinical and Pathology Data of ACTH-Treated Animals.—Except for a noticeable increase in placidity and docility, observed generally by the third injection, the ACTH-treated animals appeared normal and did not exhibit hyperpyrexia. A moderate weight gain and the absence of edema at autopsy indicated that they had not experienced excessive water retention. The thymus was somewhat reduced in size, and histologic examination revealed some loss of thymocytes. The lymph nodes and spleen (as well as other organs) appeared essentially normal. At the time of sacrifice (which was either one or two days after the last dose), their total white blood cell, differential, eosinophil, and platelet counts as well as VPRC were within the normal range. The influence of the ACTH-treatment on the adrenals will be discussed presently (Table I).

Body Weight and Adrenal Data.—In contrast to the ACTH-treated calves and the untreated controls, all of which exhibited a progressive weight increase during the experimental periods, each irradiated animal experienced a weight loss as early as one day after exposure and a consistently larger one until the day of exitus or sacrifice (Table I).

TABLE I. BODY WEIGHT AND ADRENAL WEIGHT, WATER, AND NITROGEN CONTENTS OF CONTROL, IRRADIATED, AND ACTH-TREATED CALVES

NO. AND TREATMENT OF CALF		BODY WT. (KG.) START	BODY WT. (KG.) END	DURATION OF EXPER. PERIOD BEFORE ADRENALECTOMY (DAYS)	ADRENAL GLANDS* WET WT. (GM.)	H_2O (%)	N (%)	N (GM.)	ADRENAL WT./BODY WT. AMT. (MG./KG.)	ADRENAL WT./BODY WT. INCR.† (%)	ADRENAL N/BODY WT. AMT. (MG./10 KG.)	ADRENAL N/BODY WT. INCR.† (%)
UNTREATED (CONTROLS)	1	110	119	10	6.43	79.9	9.9	0.13	54		11	
	2	115	119	12	6.96	80.1	10.1	0.14	58		12	
	3	124	128	12	6.77	79.5	9.8	0.14	53		11	
	4	126	132	11	7.20	80.0	10.1	0.15	55	(54 ± 2.6)†	11	(11 ± 0.2)†
	5	128	136	12	6.93	80.0	10.2	0.14	51		10	
	6	134	142	11	7.77	81.2	10.4	0.15	55		11	
	7	135	147	10	8.41	81.3	10.2	0.16	57		11	
	8	150	160	11	7.94	79.7	9.8	0.16	50		10	
IRRADIATED‡ (600 r WHOLE-BODY γ-RADIATION)	1	109	100	10	10.96	83.5	9.6	0.18	110	104	18	64
	2	115	97	11	7.42	80.4	9.7	0.14	77	43	14	27
	3	119	108	12	10.94	81.3	10.3	0.21	101	87	20	82
	4	122	104	12	12.37	83.5	10.0	0.20	119	120	19	73
	5	122	102	10	10.08	82.3	9.7	0.17	99	83	17	55
	6	134	115	11	13.75	82.7	9.7	0.23	120	122	20	82
	7	136	116	12	14.69	82.0	9.4	0.25	127	135	22	100
	8	142	129	11	11.50	82.8	9.1	0.18	89	65	14	27
	9	149	132	10	11.03	81.4	9.9	0.20	84	56	15	36
	10	155	115	11	14.10	83.2	8.8	0.21	123	128	18	64
ACTH I.M.§ (150 I.U./100 KG. DAILY × 8)	1	98	106	9	6.87	80.0	10.2	0.14	65	21	13	18
	2	104	118	9	12.75	80.5	9.9	0.25	108	100	21	91
	3	111	118	9	11.35	79.8	10.2	0.23	96	78	20	82
	4	119	126	8	13.11	81.2	10.1	0.25	104	93	20	82
	5	136	148	8	14.32	80.1	9.8	0.28	97	80	19	73
	6	139	151	9	10.92	79.9	9.9	0.22	72	33	15	36
	7	142	155	8	15.52	80.0	9.7	0.30	100	85	19	73
	8	149	150	9	14.49	81.0	10.3	0.28	97	80	19	73

*The values shown below represent the combined wet weight of the 2 glands from each calf, the per cent of water of the wet weight, the per cent of nitrogen of the dry weight, and the total amount of adrenal nitrogen in each animal.

†Per cent of increase in the ratio of the total adrenal weight or total adrenal nitrogen to the body weight compared to the mean control ratios of 54 (±s.d.) and 11 (±s.d.), respectively.

‡The dose employed represents the $LD_{50/9}$ or the $LD_{100/14}$.

§Each calf received 8 daily I.M. injections of 150 I.U./100 Kg. of ACTH. The first and last doses were Acthar (dissolved in approx. 10 ml. 0.9 per cent NaCl), and the 6 others were purified corticotrophin in 20 per cent gelatin.

Despite this appreciable loss in body weight, the adrenals of the irradiated animals were markedly enlarged as compared to those of the untreated animals, and exhibited absolute weight gains comparable in magnitude to those of the ACTH-treated ones. Table I indicates that the percentage water of the wet weight was somewhat higher in all but 3 pairs of the irradiated adrenals than the controls values, and that the percentage nitrogen of the dry weight was lower than normal in at least 3 pairs. It should be added that pairs 1, 4, and 10—the ones with the highest percentage water—were all appreciably hemorrhagic. While undoubtedly some of the weight gain of the irradiated adrenals was attributable to edema, most of it was due to the formation of new protoplasmic constituents as evidenced by the total adrenal-nitrogen values. Regarding the ACTH-treated adrenals, their percentage water and nitrogen were comparable to those of the untreated controls; hence, their weight gain was associated solely with a corresponding increment in protoplasmic components.

The mean values of the adrenal weight/body weight ratios of the control, irradiated, and ACTH-treated calves were 54, 105, and 92 mg./Kg., respectively. The corresponding values of the adrenal-nitrogen/body weight ratios were 11, 18, and 18 mg./Kg., respectively.

Adrenal Perfusion Data.—Table II summarizes the analytical data of studies in which each pair of adrenals (shown in Table I), excised from untreated, ACTH-treated, or irradiated calves in extremis, was perfused for two hours at 37.5° C. with a liter of a well-oxygenated artificial medium, supplemented with 10 I.U./hr. of ACTH. These experiments were designed to ascertain the overall or integrated influence of the many pathologic alterations contributing to radiation sickness death on the functional status of the adrenal cortex at the time of death, as evidenced by the nature and quantity of its steroidal secretory output in response to maximal corticotrophic stimulation. Inasmuch as the glands, removed terminally from the irradiated animals, possessed markedly enlarged cortices,* it was deemed desirable to compare their steroid-producing capacity not only to control glands but also to adrenals from ACTH-treated animals of comparable age and weight.

Regarding the influence of radiation sickness on the nature of the adrenocortical secretory product at the time of death, Table II indicates that the terminally removed adrenals biosynthesized and released the same corticosteroids as glands from either the control or ACTH-treated animals; namely, cortisol (F), cortisone (E), and corticosterone (B).† Aside from large amounts of these quantitatively major corticosteroids, in each case the chromatograms revealed minute quantities of an aldosterone-like material[15] and an "X" zone. The latter constituted a comparable percentage (approximately 11 per cent) of the total corticoid output of the exposed, untreated, and ACTH-treated glands, and comprised unidentified steroids more polar than F. No detectable amounts of 11-dehydrocorticosterone (A), 11-desoxycorticosterone (DOC), 11-desoxycortisol (S), or other steroids not ordinarily recovered from the control effluents were ever found in the effluents of the glands from the moribund animals despite successive

*An effect presumably attributable in the main to an enhanced production and/or release of endogenous ACTH in response to the generalized radiation damage.

†These have all been isolated in crystalline form from their respective chromatographic zones and found to be identical to authentic samples by melting point and infrared analysis.[11]

resolutions in the toluene-propylene glycol or ligroin-propylene glycol chromatographic systems. Thus, adrenocortical dysgenesis did not occur despite the varied pathologic derangements contributing to the radiation sickness death.

However, there seemed to be a significant qualitative change in the terminal pattern of biogenesis with respect to the relative activity of the 17-alpha hydroxylating system. Of the total corticoid outputs (F, E, and B) secreted by the irradiated and control glands, the 17-hydroxylated portions (F and E) constituted 85 ± 0.9* and 75 ± 1.7* per cent, respectively. In this regard, it is

TABLE II. THE CORTICOID OUTPUT OF PERFUSED PAIRS OF ACTH-STIMULATED ADRENALS* EXCISED FROM UNTREATED AND ACTH-TREATED CALVES, AND FROM IRRADIATED CALVES NEAR DEATH†

NO. AND TREATMENT OF CALF		MAJOR CORTICOIDS FOUND IN PAPER CHROMATOGRAPHIC ZONES‡			OUTPUT OF F, E, AND B	APPARENT CONDITION AT SACRIFICE	
		F (μ)	E (μ)	B (μ)	(μ)	CALF	ADRENALS
UNTREATED	1	895	180	375	1,450	Normal	Normal
	2	881	175	333	1,389	Normal	Normal
	3	767	157	326	1,250	Normal	Normal
	4	894	185	247	1,326	Normal	Normal
	5	1,104	287	289	1,680	Normal	Normal
	6	721	179	445	1,345	Normal	Normal
	7	1,190	218	423	1,831	Normal	Normal
	8	791	190	393	1,374	Normal	Normal
IRRADIATED	1	1,070	156	261	1,487	Moribund (C)†	Hypertr. and hemor.
	2	1,658	280	360	2,298	? Moribund (W)†	Hypertr.
	3	1,877	368	326	2,571	? Moribund (W)	Hypertr.
	4	698	192	129	1,019	Died	Hypertr. and hemor.
	5	1,520	222	426	2,168	Moribund (C)	Hypertr.
	6	1,026	155	208	1,389	Moribund (C)	Hypertr. and hemor.
	7	1,204	292	151	1,647	Died	Hypertr.
	8	835	88	186	1,109	Moribund (C)	Hypertr.
	9	1,990	254	346	2,590	Moribund (C)	Hypertr.
	10	699	129	143	971	Moribund (C)	Hypertr. and hemor.
ACTH-TREATED	1	2,605	318	525	3,448	Placid and docile	Normal
	2	4,610	596	510	5,716	Placid and docile	Hypertr.
	3	3,812	337	633	4,782	Placid and docile	Hypertr.
	4	5,784	511	492	6,787	Placid and docile	Hypertr.
	5	5,927	704	604	7,235	Placid and docile	Hypertr.
	6	4,002	553	524	5,079	Placid and docile	Hypertr.
	7	6,869	792	982	8,643	Placid and docile	Hypertr.
	8	6,201	1,225	532	7,958	Placid and docile	Hypertr.

*The 2 adrenals of each calf shown in Table I were perfused in parallel for 2 hours at 37.5° C. with a liter of a well-oxygenated artificial medium, to which 10 I.U./hr. of ACTH was added for maximal stimulation.

†The adrenals were excised from the irradiated animals immediately after spontaneous death or when they appeared to be in extremis. The "C" in parentheses refers to those irradiated animals which were unable to walk and were carried to the slaughter site; the "W," to those which walked.

‡Zones F (cortisol) and E (cortisone) were measured by the Porter and Silber method; zone B (corticosterone) by the phosphomolybdic acid reaction. All 3 assays were confirmed by reaction with alkaline blue tetrazolium.

noteworthy that the adrenals from the ACTH-treated animals also secreted a significantly greater proportion of 17-hydroxylated corticoids (90 ± 1.0 per cent) than did those from untreated animals.

*The standard error of the mean percentages of 17-hydroxylated corticoids.

Table II also illustrates the total 2-hour corticoid outputs of the in vitro perfused pairs of adrenals, excised from the individual animals and stimulated maximally with ACTH. The mean outputs of the glands from the control, exposed, and ACTH-treated calves were 1,456, 1,725, and 6,206 μg, respectively. Omitting the data of the 2 irradiated calves (2 and 3) which walked to slaughter and probably were not in a truly moribund condition, as previously defined, the mean corticoid output of the exposed adrenals was 1,548 μg. In contrast to the controls, considerable variability will be noted among the outputs of the irradiated glands. Nevertheless, it is striking to find that the mean corticoid output of adrenals obtained from animals at or on the verge of death following a lethal radiation dose is comparable to that of normal glands. Even in the case of those exposed adrenals (1, 4, 6, and 10), which were friable, edematous, and hemorrhagic, the mean 2-hour steroid-producing capacity (1,217) was not strikingly lower than that of the control glands (1,456). Thus, a comparison of only the pre-exposure or normal and postexposure terminal outputs would permit the conclusion that, in general, the adrenal glands were "holding their own" at the time of death from acute radiation sickness. To be sure, if their corticoid outputs are compared with those from glands which were also enlarged and of similar size, i.e., glands from the ACTH-treated animals, a remarkable functional depletion is revealed.

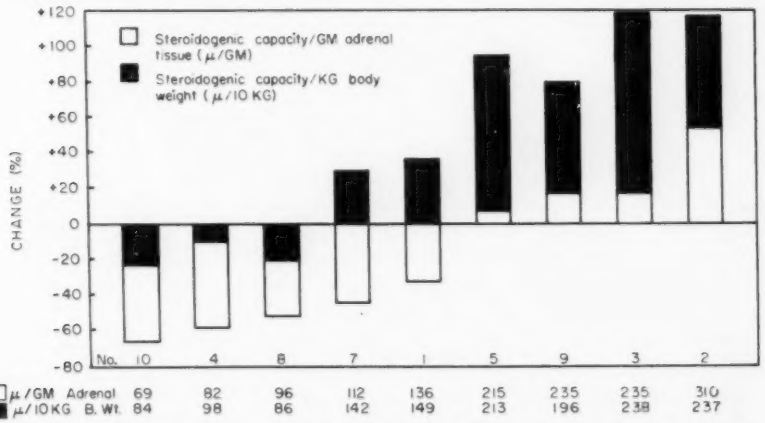

Fig. 1.—The alterations in the terminal adrenocortical function of calves dying from acute radiation sickness. The open bar represents the per cent increase (+) or decrease (−) in the steroidogenic capacity per gram of adrenal tissue compared to the mean control ratio of 200 (±3.5, the s.e. of the per cent difference from the mean). The solid bar represents the per cent increase (+) or decrease (−) in the steriodogenic capacity per 10 kilograms of body weight compared to the mean control ratio of 109 (±4.7, the s.e. of the per cent difference from the mean). Unlike the others, animals 2 and 3 were able to walk and probably were not really terminal when sacrificed.

In view of the substantial increase in the adrenal weight and the concomitant decrease in the body weight of the irradiated calves, it would be more appropriate to review the corticoid output data of Table II in terms of the corresponding adrenal and body weights of the various animals, as shown in Table III. As previously mentioned, the corticoid outputs (Table III) indicate that the irradiated gland outputs per gram of adrenal tissue or per kilogram body weight

TABLE III. THE COMPARATIVE STEROIDOGENIC CAPACITY* OF PERFUSED PAIRS OF ACTH-STIMULATED ADRENALS EXCISED FROM UNTREATED AND ACTH-TREATED CALVES, AND FROM IRRADIATED CALVES NEAR DEATH†

	NO. AND TREATMENT OF CALF	COMBINED WT. OF LEFT AND RIGHT ADRENALS (GM.)	BODY WT. AT SACRIF. (KG.)	OVER-ALL STEROIDOGENIC CAPACITY*				
				MAX. OUTPUT (M.O.) (μ)	M.O./GM. ADRENALS (μ/GM.)	CHANGE‡ (PER CENT)	M.O./KG. BODY WT. (μ/10 KG.)	CHANGE‡ (PER CENT)
UNTREATED	1	6.43	119	1,450	226		122	
	2	6.96	119	1,389	200		117	
	3	6.77	128	1,250	185		98	
	4	7.20	132	1,326	184	(200 ± 3.5)‡	101	(109 ± 4.7)‡
	5	6.93	136	1,680	242		124	
	6	7.77	142	1,345	173		95	
	7	8.41	147	1,831	218		125	
	8	7.94	160	1,374	173		86	
IRRADIATED	1	10.96	100	1,487	136	−32	149	+37
	2	7.42	97	2,298	310	+55§	237	+117§
	3	10.94	108	2,571	235	+18§	238	+118§
	4	12.37	104	1,019	82	−59	98	−10
	5	10.08	102	2,168	215	+8	213	+95
	6	13.75	115	1,389	101	−50	121	+11
	7	14.69	116	1,647	112	−44	142	+30
	8	11.50	129	1,109	96	−52	86	−21
	9	11.03	132	2,590	235	+18	196	+80
	10	14.10	115	971	69	−66	84	−23
ACTH-TREATED	1	6.87	106	3,448	502	+151	325	+198
	2	12.75	118	5,716	448	+124	485	+345
	3	11.35	118	4,782	421	+111	405	+272
	4	13.11	126	6,787	518	+159	539	+395
	5	14.32	148	7,235	505	+153	489	+349
	6	10.92	151	5,079	465	+132	336	+208
	7	15.52	155	8,643	557	+179	558	+412
	8	14.49	150	7,958	549	+175	531	+387

*The steroidogenic capacity has been expressed in 3 ways: (a) the maximum output (M.O.) or total 2-hour output of F, E, and B, (b) the maximum corticoid output per gram of adrenal tissue, and (c) the maximum total corticoid output per kilogram of body weight at sacrifice.

†The adrenals were excised from the exposed animals immediately after spontaneous death or when they appeared to be in extremis.

‡The change refers to the per cent increase (+) or decrease (−) in the steroidogenic capacity per gram of adrenal tissue or per 10 kilogram of body weight compared to the mean control ratios of 200 (±3.5, the s.e. of the per cent difference from the mean) and 109 (±4.7, the s.e. of the per cent difference from the mean), respectively.

§Although these animals (2 and 3) were diagnosed as moribund at the time of sacrifice, it is noteworthy, in view of the analytical findings, that they were the only ones able to walk to the slaughter site.

varied considerably in contrast to the moderate variability in the control (and ACTH) data. The mean microgram output per gram of adrenal tissue (and the range) of the exposed, control, and ACTH-treated groups were as follows: 159 (69 to 310), 200 (173 to 242), and 496 (421 to 557). The mean microgram output per 10 kilogram of body weight (and the range) of these 3 groups, in the same order, were as follows: 156 (84 to 238), 109 (86 to 125), and 459 (325 to 558).

Whereas the steroidogenic capacity per gram of adrenal tissue was markedly depleted in 6 of the 10 animals dying of acute radiation sickness, it was of similar magnitude in 3 and significantly greater than normal in 1 (No. 2, probably not a terminal animal). In striking contrast to these findings, the steroidogenic capacity per kilogram of body weight was only slightly depleted in 3 of these agonal animals, was moderately above normal in 3, and significantly greater than normal in the remaining 4. To summarize, the mean terminal steroidogenic capacity of the irradiated animals was 35 per cent* below normal on the basis of their adrenal tissue and 25 per cent* above normal on the basis of their body weight. The individual alterations in the adrenocortical function of the calves dying of acute radiation sickness are graphically shown in Fig. 1, and are based on the control data of Table III. Comparison with the ACTH data of Table III would reveal remarkable depletions in steroid-producing capacity on either an adrenal or body weight basis.

DISCUSSION

Extensive studies involving a similar experimental design were previously conducted by the author[16] in order to ascertain the alterations in the adrenocortical function of calves during the first 9 days after exposure to the same dose (600 r) of whole-body irradiation presently employed. It was observed that the total 2-hour corticoid output as well as the output per gram of adrenal tissue increased substantially within 24 hours after exposure. After both had declined to normal or slightly subnormal levels by the sixth day, an appreciable secondary or preterminal rise occurred. Following this preterminal peak in the steroid-producing capacity, a marked decline to subnormal levels was noted by the ninth day only in those animals which were patently in very bad condition. However, the calves which were sacrificed on the ninth day in apparently good condition exhibited a total output or output per gram of adrenal tissue which was substantially elevated. In this connection, it must be emphasized that the condition of the calves varied considerably after the seventh or eighth day. Obviously the striking difference observed in the latter 2 groups on the ninth day was referable to the fact that the former group was preterminal and the latter essentially a terminal one.

In the present studies, a truly agonal state or death was used as the end point in order to evaluate the nature and extent of the terminal depletion in the steroidogenic capacity of the cortex under extremely adverse conditions. The results indicated that adrenocortical dysgenesis did not occur despite the development of generalized sepsis, toxemia, hemorrhage, anemia, and other

*Animals 2 and 3 were not included in these calculations because they probably were not truly terminal animals.

pathologic sequelae associated with the radiation sickness syndrome. The relative increase in the activity of the 17-alpha hydroxylating system, observed in the adrenals from the ACTH-treated* animals, was also found in the terminally-removed, irradiated glands. Perhaps, this biogenetic shift in the exposed glands is also related to the in vivo influence of ACTH, released endogenously in greater amounts in response to the generalized radiation damage. Aside from the absence of dysgenesis, it was interesting to find that the irradiated glands were "holding their own" at the time of death. Whereas their mean capacity per gram of tissue was depleted 35 per cent, their mean maximum output was slightly higher (6 per cent) and their capacity per kilogram of body weight 25 per cent higher than before exposure.

Since not only the total amount of cortical tissue† but also its proportion of the whole gland increased following the exposure as a result of the differential, growth-promoting action of endogenously-released ACTH, it was of interest to compare the steroidogenic capacity per gram of adrenal tissue and per kilogram of body weight of glands from moribund calves with the functional capacity of adrenals of comparable size, obtained from animals pretreated with ACTH. In this manner, it was hoped to evaluate the intrinsic damage to the adrenal cortex due to acute radiation sickness, and to dissociate the indirect (pituitary-mediated), growth-promoting influence of irradiation from its primary and secondary deleterious effects. Obviously, adrenocortical alterations, provoked solely by increased levels of ACTH (as in the case of the ACTH-treated animals), would not be associated with the probable inhibitory and/or destructive effects incident to the host of radiation-induced derangements. A comparison of the ACTH and control data of Table III provides an estimate of the functional change which would have occurred in the irradiated glands in concomitance with their substantial growth if the proliferative influence of endogenously-released ACTH had not been accompanied by any toxic effects. Subsequent comparison of the ACTH and irradiation data affords considerable insight not only into the extent of the intrinsically destructive influence of irradiation but also regarding the magnitude of the resulting adrenocortical compensation. Although the mean steroidogenic capacity of the irradiated animals was only 35 per cent below normal on the basis of their adrenal tissue and 25 per cent above normal on the basis of their body weight, it was 74 and 70 per cent, respectively, below the mean steroid-producing capacity of the ACTH-treated animals.

However, to conclude from the latter comparison that the irradiated glands were exhausted would be improper and misleading inasmuch as the adrenals from the ACTH-treated animals were not normal organs but hyperfunctional ones. Nor does comparison with the control organs properly permit this conclusion in view of the fact that the terminal response of the irradiated glands to maximal stimulation, albeit not commensurate with their size, at least was comparable to their pre-exposure response. Parenthetically, it would appear to

*This in vivo action of ACTH should be differentiated from a similar but acute in vitro effect. When contralateral adrenals are perfused in vitro with and without ACTH, the stimulated ones biosynthesize a greater proportion of 17-alpha hydroxycorticoids than the unstimulated controls.[7]

†The cortical portion of the normal calf adrenal constitutes approximately 70 per cent of the wet weight of the whole gland.[7]

the author that the meaning of the phrase "adrenal exhaustion," as it usually appears in the literature, is often inaccurate or misinterpreted and that this terminology should not be applied unless it has been demonstrated, as in Addison's disease, that the cortex possesses a decisively diminished capacity to elaborate the cortical hormones. Even its use in the Waterhouse-Friderichsen syndrome, which is characterized by massive bilateral adrenal hemorrhage, is misleading because it suggests a process of attrition rather than the abrupt necrosis and cessation of function which occurs. Despite the varied histologic and histochemical alterations in the adrenals which have been observed by many investigators during the so-called "exhaustion stage" of the general adaptation syndrome[1] and considered to be pathologic, it is the function and capacity of the cortex which is of critical importance and not its appearance or contents. In fact, studies now in progress to correlate the functional status of the terminally-removed adrenals with their histologic and histochemical characteristics have already indicated that such commonly employed indices of adrenocortical function as sudanophilia and birefringence may be unreliable or false.

It should be emphasized that the present findings per se, while demonstrating that the terminal function of the cortex is far from exhausted, provide little information as to the relative hormonal needs of the dying animals and to the possibility of an actual insufficiency of the adrenal steroids preceding and contributing to their death. In this connection, the data of a report to be published, concerning the peripheral blood corticoid levels following irradiation, clearly show that during the preterminal period when the steroid-producing capacity begins to decline, there is an actual increase in the level of circulating corticoids, and that on the day of death the blood level is strikingly elevated. In fact, the increase is significantly larger than can be observed at any time in animals given a massive dose of ACTH.* Space limitations do not permit a review of the voluminous and conflicting literature describing attempts to influence irradiation survival time or mortality with adrenal cortical hormones. However, from the foregoing it would not appear beneficial to administer supplementary amounts of corticosteroids to a preterminal or terminal animal, in which the endogenously-secreted adrenal steroids are already accumulating (because of a failure in their utilization and/or excretion) and possibly exerting damaging effects.

SUMMARY

In order to evaluate the functional status of the adrenal cortex at the time of death from a fatal stressor, the adrenals of intact calves, exposed to a lethal dose of whole-body gamma radiation, were removed immediately before death. They were then perfused with a well-oxygenated artificial medium to which ACTH was added in order to ascertain the nature and quantity of their corticosteroidal output in response to maximal stimulation. In view of their marked hypertrophy, the glands were compared not only to control adrenals but also to glands of comparable size obtained from calves pretreated with ACTH.

*The peripheral corticoid blood levels of the dying animals are frequently higher than even those observed in animals given a massive dose of ACTH after 8 daily pretreatment doses.

Whereas the 17-hydroxylated portion of the total corticoids biosynthesized by the irradiated adrenals was moderately increased, the results indicated that a terminal adrenocortical dysgenesis did not occur despite the development of generalized sepsis, toxemia, hemorrhage, anemia, and other pathologic sequelae associated with the radiation sickness syndrome. It was also interesting to find that the irradiated glands were "holding their own" at the time of death. Although their mean capacity per gram of tissue was depleted 35 per cent, their mean maximum output was slightly higher (6 per cent) and their capacity per kilogram of body weight was 25 per cent higher than before exposure. Comparison with the adrenals from the ACTH-treated animals, however, indicated that their steroidogenic capacity was not commensurate with their substantial growth, and afforded considerable insight not only into the extent of the intrinsically destructive influence of irradiation but also regarding the magnitude of the resulting adrenocortical compensation.

The findings strongly suggest that the terms "adrenal exhaustion" and "dysgenesis" may be misleading or inaccurate as presently applied in a variety of clinical conditions associated with irradiation, infections, intoxications, burns, and other noxious agents.

The able technical assistance of N. Du Bois and J. W. Goslin is gratefully acknowledged by the author.

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CONSTANCY OF URINARY CREATININE EXCRETION

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INTRODUCTION

THIS investigation was undertaken in an attempt to determine the constancy of creatinine excretion in the urine of normal persons under everyday non-regulated conditions.

It has been a generally accepted assumption for many years that creatinine excretion in the urine can be used as a check for the completeness of urine collection. One of the more widely used reference works in biochemistry[1] states: "In the adult male it was shown . . . the daily excretion of creatinine is constant over long periods, not only under standard conditions, but under the most varied circumstances. It is little affected by diet, exercise or large variations of urine volume. Apparently the creatinine excretion is characteristic for a given individual in health and is determined chiefly by his size. Because of this relationship the daily excretion of creatinine is often used to check the accuracy of 24-hour urine collections."

This constancy of creatinine excretion in 24-hour periods has been assumed by a number of investigators to be true for short term collection periods. In addition, creatinine excretion has been frequently used for the formation of ratios; for example, uric acid/creatinine ratio, 17-ketosteroid/creatinine ratio, catechol/creatinine ratio, etc. The advantage of such ratios when used to judge the effect of stimuli on the numerator of the ratios (assuming a constant creatinine excretion) is that the effect of incompleteness of urine collection or inaccuracy in the timing of collection periods would be minimized. The assumption that creatinine excretion is constant in short collection periods is based mainly on the work of Shaffer[2] quoted by Peters and Van Slyke. A remarkably constant hourly excretion was demonstrated in 3 persons followed at 1- to 5-hour intervals during several 24-hour periods and in 4 persons on whom 2 or 3 collections were made for different periods within a single day.

However, a perusal of the literature concerning constancy of creatinine excretion shows that although other investigators found results similar to Shaffer's, there were also studies[3] that showed considerable variability in short term collection periods. It is, however, clear that none of these investigators had sufficient data to permit generalizations about creatinine excretion.

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The investigation was supported in part by Research Grant (M-855) from the National Institute of Mental Health of the National Institutes of Health of the U. S. Public Health Service, Bethesda, Md., and grateful acknowledgment is made to Eli Lilly & Company for its aid and support in carrying out this investigation.

Received for publication June 20, 1957.

Therefore, we decided to collect more comprehensive data in an attempt to answer our questions: How good is the determination of the excretion of creatinine in the urine as a check on the accuracy of 24-hour urine collections? Is it justifiable to use creatinine excretion as a check on the accuracy of short term collections or for incorporation into ratios?

MATERIALS AND METHODS

Estimation of Creatinine.—A micromodification of the Folin[4] method has been used. As has been shown in several studies[5, 6] the easily performed direct Folin method when applied to urine will give results that are very close to those obtained with more complicated methods using either Lloyd's reagent or creatinine-destroying bacteria to improve the specificity of the method.

The micromodification was done using Krogh-Key's syringe pipettes since they are faster and more accurate than ordinary volumetric pipettes.[7] A 1.0 N NaOH picric acid solution (1.2 ml. of freshly prepared one part NaOH solution to 5 parts saturated picric acid mixture) was added with a syringe pipette to the urine (0.05 ml. measured out with a constriction pipette). This mixture was allowed to stand for exactly 10 minutes and then diluted with distilled water (8.75 ml. added with a syringe pipette). The readings were done at accurately timed intervals on a Beckman model DU spectrophotometer, at a wave length of 480 mμ. Creatinine standards and reagent blanks were run with each set of analyses. Standards and urines followed Beer's law when read against a reagent blank at the wave length chosen.

This micromodification when compared with Folin's method was found in stop watch studies with experienced personnel to be faster by a factor of two. To test the accuracy of the method, 12 analyses were done on a pooled urine. The concentration was found to be 1.58 Gm. creatinine/liter with a standard deviation of 0.010 which corresponds to a coefficient of variation of 0.62 per cent.

All pronounced variations in creatinine excretion were rechecked with a second set of analyses.

The Subjects.—The persons (ten women, eight men) collecting urine for these investigations were employees of the Research Facility and Rockland State Hospital. They were in good health at the time of the investigation, and their ages varied between 19 and 45 years. The women were all in the nonmenstruating phase.

Organization of the Study.—The subjects led normal lives. They were, however, asked to write down at what time meals were taken and the quantities of the different foods they had eaten.

Urine was collected in 2-hour periods from 8 A.M. until 10 P.M. and one night portion from 10 P.M. until 8 A.M. Instructions were given to the subjects to make sure that each collection was complete. Although the collection days were usually consecutive days within the week, this was not possible with each participant in the collections.

RESULTS

We have summarized our findings of the variability of creatinine excretion in Table I. The total excretion per 24 hours has been calculated by adding up the seven 2-hour periods and the one 10-hour night period to give the sum for the 24 hours. The standard deviation for the ten 24-hour periods has then been calculated to give a measure of the spread of the data for each individual person. The coefficient of variation in per cent, which is the standard deviation divided by the mean excretion expressed in per cent, has been calculated in all cases to make comparison between the different individuals easier.

Fig. 1 shows the findings for the short collection periods. Each narrow bar represents a two-hour collection period; the broad-based bars represent the night periods.

TABLE I

WOMEN					MEN				
INITIALS	AGE	MEAN EXCRETION OF CREATININE PER 24 HOURS (GM.)	STANDARD DEVIATION	COEFFICIENT OF VARIATION (PER CENT)	INITIALS	AGE	MEAN EXCRETION OF CREATININE PER 24 HOURS (GM.)	STANDARD DEVIATION	COEFFICIENT OF VARIATION (PER CENT)
J. A.	36	1.14	0.06	5.2	N. A.	19	1.72	0.12	7.0
M. F.	35	1.07	0.03	2.8	W. C.	43	1.83	0.27	14.6
R. L.	25	1.36	0.04	2.9	W. D.	41	2.35	0.04	1.7
J. L.	25	1.12	0.08	7.4	T. L.	35	1.75	0.09	5.2
F. M.	31	1.16	0.14	12.0	I. P.	25	2.20	0.24	10.8
C. P.	45	1.20	0.02	1.8	H. P.	44	1.60	0.13	8.1
B. R.	22	1.20	0.12	10.0	B. S.	35	1.21	0.21	17.3
M. S.	33	1.16	0.07	6.0	P. V.	36	1.77	0.16	9.0
D. S.	22	1.15	0.04	3.8					
M. T.	35	1.12	0.03	2.8					
Mean:	31	1.17			Mean:	35	1.80		
S.D.		0.08			S.D.		0.35		

Mean excretion of creatinine per 24 hours for 10 normal women and 8 normal men. Each of the values listed is the average of 10 determinations except for W. D. (6) and N. A. (9). The standard deviations and coefficients of variations have been calculated for each person to show the great individual differences in the spread of the data.

DISCUSSION

Constancy of Creatinine Excretion in 24-Hour Periods.—Table I shows that there are very large individual variations in variability of creatinine excretion in the 24-hour periods and that the determination of creatinine as a check on completeness of urine collection must be rated all the way from excellent to poor, depending upon the individual under investigation.

For persons like M. F., R. L., C. P., M. T., and W. D. there is a better than 1 in 100 probability that daily creatinine values will vary no more than 7.5 per cent around the mean. Therefore, in these subjects creatinine excretions seem to be an excellent check on the completeness of urine collection. But in persons like F. M., W. C., and B. S. the check for completeness would be quite unreliable since variations greater than 20 per cent around the mean could be expected to occur 1 time out of 10. Consequently, in any random selection of subjects for an experimental study there is considerable risk of including persons with high variability in creatinine excretion. For example, in our study, 4 out of 18 persons have shown coefficients of variation greater than 10 per cent.

When creatinine excretion is planned to be used as a check on completeness of urine collection in 24-hour periods, it would be best first to determine the variability of the excretion in the individuals under investigation. Then the determination of creatinine excretion per 24 hours would be a valuable research tool. However, the use of creatinine excretion per 24 hours as a check on urine collection cannot be recommended on randomly selected individuals, whose variability of creatinine excretion is not known.

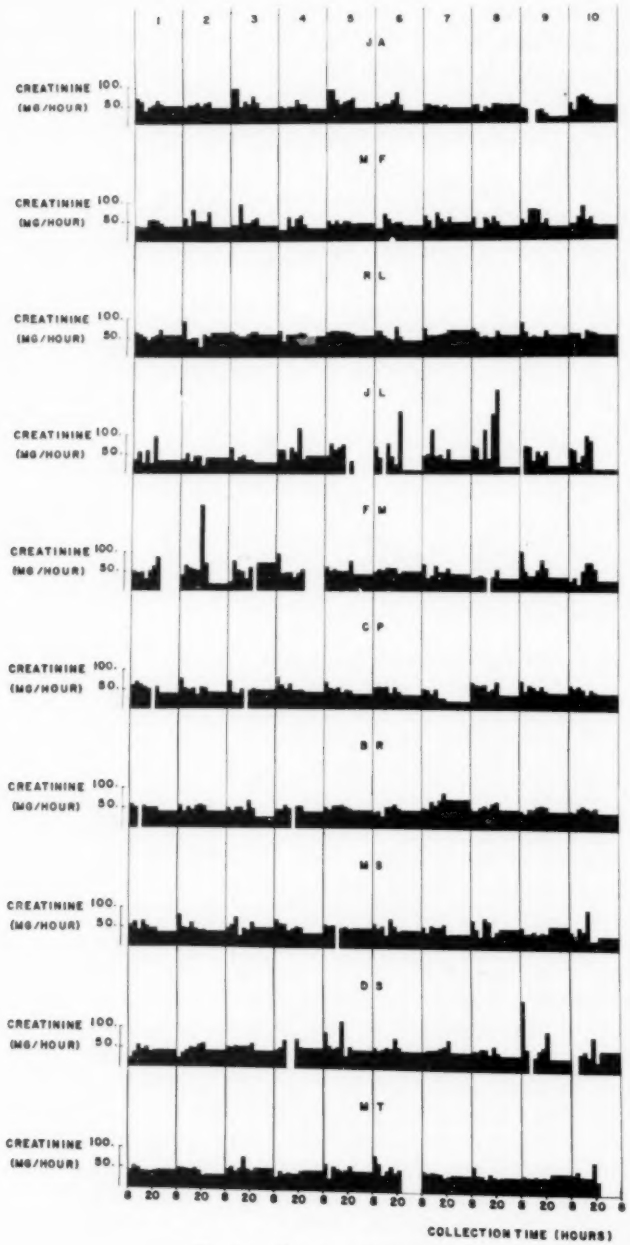

Fig. 1.—*(For legend see opposite page.)*

Constancy of Creatinine Excretion in Short Collection Periods.—Here too, but accentuated, the same picture is found as for the 24-hour collection periods. The usefulness of creatinine excretion for checking purposes in short collection periods and in ratios is entirely dependent on the individual under investigation.

As Fig. 1 shows, the measurement of creatinine excretion could be used in short collection periods as a check on the completeness of collection of urine in the persons R. L., B. R., and M. T.

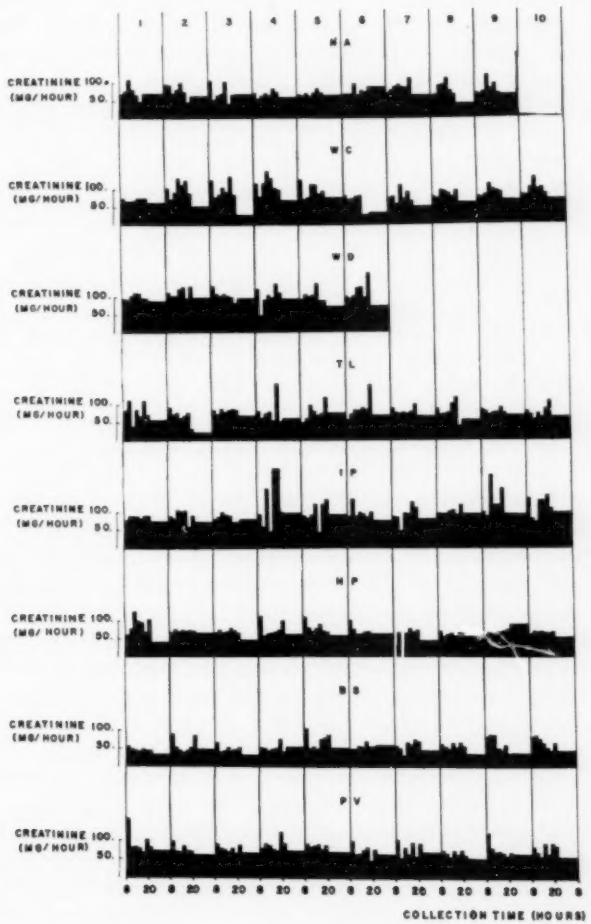

Fig. 1.—Creatinine excretion data for short collection periods. Data from 10 women (*left*) and 8 men (*right*). Urine collected in seven 2-hour periods from 8 A.M. until 10 P.M. and in one 10-hour night portion for a total of 10 days on each person except for N. A. (9 days) and W. D. (6 days). Excretion of creatinine in milligrams/hour on the ordinate. Time in hours on abscissa.

However, the use of creatinine excretion for checking purposes in some of the persons with a very high variability in excretion like W. C. or J. L. is obviously not justified since the variation from hour to hour will frequently exceed 100 per cent.

It is clear that creatinine excretion should not be used for checking purposes in short collection periods or incorporated in ratios unless a preliminary study of variability in excretion is done on the individual.

Other Observations.—Our study confirms that men as a group excrete more creatinine than women, the mean for men being 1.80 Gm./24 hours and for women 1.17 Gm./24 hours (a difference that is significant at better than the 0.01 probability level), in agreement with the theory proposed by Shaffer[2] and modified by Myers and Fine[8] of a constant relation between amount of creatine in the muscles and creatinine excretion. It also confirms other authors' findings that creatinine excretion is lowest at night.[3, 9]

Perhaps the most interesting question this study raises is, "Why is there in some persons such pronounced variability in creatinine excretion?"

The possibility of analytical errors can be ruled out. With a coefficient of variation for the analytical method of less than 1 per cent, analytical errors cannot be expected to account for more than maximally 2 to 3 per cent of some of the larger variations.

The possibility does of course exist that some of the persons investigated are not able to empty their bladder completely at 2-hour intervals and at one time will empty more completely than at another time. P. V. has previously, in repeated self-experiments, found that the amount of urine in his bladder on catheterization was no greater than what could be calculated to enter the bladder through the ureters during the time it took to do the catheterization and wash out the bladder with distilled water. This subject, although having one of the more stable excretion patterns, occasionally showed rather pronounced spontaneous variations from 2-hour period to 2-hour period. In one case, a drop to 50 per cent of the value from the period immediately preceding was observed, in another an increase of 80 per cent. In this case it seems almost certain that the emptying of the bladder was complete and that his variability in excretion must be accounted for in another way. This does not mean that other persons could not vary in the degree in which they empty their bladder. A systematic study of this factor in all the subjects has not been possible.

The question as to what the effect of ingestion of meat might have, especially in short term experiments, is an important one since meat is the main creatine source in food and any transformation of creatine to creatinine during preparation of food or during digestion could be expected to be reflected in increased creatinine excretion in the urine. Klercker[10] did a systematic investigation of this problem and found no change either in short term experiments or in 24-hour samples after ingestion of meat from different species in amounts up to 800 Gm. per portion. Our data support these findings of Klercker. The subjects under investigation by us kept notes of approximated quantities of

food taken at meals and at what time meals were taken and no correlation between meat intake and increases in creatinine excretion can be noticed in our material. Meat extract presents a special problem since it contains considerable amounts of creatinine. No meat extracts were used in the preparation of meals for these subjects, however, and canned soups were taken only once or twice, by a few subjects, owing to the time of the year the study was done.

It is also unlikely that variations in blood creatinine could give variations as encountered here. Haugen and Blegen,[11] who have had extensive experience with estimation of creatinine in blood, state that "daily determinations of creatinine values in the single normal individual show no variations for weeks."

We would like to suggest the possibility of variation in glomerular filtration rate as a possible explanation for some of the variability in creatinine excretion. Although the glomerular filtration rate has been found[12] to be fairly constant under standard conditions, it has been shown[13] that a number of factors can effect glomerular filtration rate. In one experiment, Brod[13] reports an increase of 300 per cent in glomerular filtration rate from one 3-hour period to the next. Therefore, it seems possible that the variability in creatinine excretion in our present study could be explained on the basis of sudden changes in glomerular filtration rate. Admittedly, glomerular filtration rate would have to be much more labile in some persons than is generally assumed.

It must be conceded, however, that tubular excretion of creatinine remains a possibility that could account for all or some of the variability found; such an explanation would imply that tubular excretion in some cases would have to account for 75 per cent of the creatinine excreted, a figure far in excess of reported values in man.

SUMMARY

1. In a study of variability in 18 normal persons, the constancy of urinary excretion of creatinine in 24-hour collection periods has been found highly dependent on the subject under investigation. Great constancy has been found in some, fair constancy in most, and poor constancy in some.

2. The estimation of urinary creatinine excretion in short collection periods has been found to vary considerably more than 24-hour estimations. Creatinine should not be used to check the completeness of urine collection in short experimental periods nor should it be used for ratio formation under such conditions, unless a preliminary investigation of variability is undertaken.

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GUANIDINIUM COMPOUNDS IN BLOOD AND URINE OF PATIENTS SUFFERING FROM MUSCLE DISORDERS

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AN EXCELLENT review of creatine and creatinine metabolism and diseases of the neuromuscular system was published by Milhorat in 1953.[1] Creatinuria, decreased creatinine excretion, and a decreased ability of the body to retain ingested creatine or creatine precursors have been reported in various muscle diseases.[2-5] The normal individual can retain small doses of ingested creatine,[6-8] excretes a small amount or no creatine,[9] and has a constant excretion of creatinine per unit of body weight.[10-11] It has, therefore, been suggested that in these muscle disorders there is a decreased ability of the muscle cells to "utilize" creatine.[12, 13] It has also been reported that guanidine and methylguanidine, compounds structurally similar to creatine and creatinine, are increased above normal levels in the blood of patients with poliomyelitis and muscular dystrophy.[14]

The creatine-creatinine and guanidine-methylguanidine data previously reported have been obtained with nonspecific analytical methods.[15-16] Recently, new procedures of analysis for creatine, creatinine, arginine, guanidinoacetic acid, guanidine, and methylguanidine have been developed.[16] It seemed worth while to investigate the blood and urine levels of these compounds in various muscle disorders using the newer analytical methods and to compare the values with those of blood and urine from normal individuals.

Thirteen different muscle diseases were investigated and it was found that in 12 of the disorders there was the following: An increased urinary excretion of creatine and guanidinoacetic acid accompanied by a decreased creatinine excretion. Blood levels of creatine were increased while guanidinoacetic acid and creatinine did not change. Blood and urine arginine levels were inconsistent and showed no significant trend. No guanidine or methylguanidine was found in the blood or urine from normal people or from individuals with poliomyelitis or muscular dystrophy.

METHODS

Blood was obtained by venipuncture and placed in a tube containing potassium oxalate. No attempt was made to obtain the blood when the subject was in the fasting state. Urine was collected without catheterization with toluene added as a preservative. The blood and

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This work was supported in part by a research grant (A-883) to the University of Minnesota from the National Institute of Arthritis and Metabolic Disease of the National Institutes of Health, Public Health Service.

Received for publication June 21, 1957.

TABLE I. CREATINE, CREATININE, AND GUANIDINOACETIC ACID IN BLOOD AND URINE OF NORMAL INDIVIDUALS AND OF PATIENTS SUFFERING FROM VARIOUS MUSCLE DISORDERS

NO. OF SUBJECTS	CLASSIFICATION	SEX	AGE	URINE (MG./KG./24 HOURS)			BLOOD (MG./100 ML.)		
				CREATINE	CREATININE	GUANIDINO-ACETIC	CREATINE	CREATININE	GUANIDINO-ACETIC
40	Normal child	17 M, 23 F	1-15	—	—	—	3.4 ± 0.92	0.4 ± 0.15	< 0.3
18	Normal adult	18 M	20-30	0	22 ± 3.4	0.6 ± 0.20	2.9 ± 0.40	0.6 ± 0.14	< 0.3
20	Normal adult	10 M, 10 F	60-100	0	14 ± 3.8	0.7 ± 0.22	3.0 ± 0.75	0.8 ± 0.24	< 0.3
5	Muscular dystrophy, pseudohypertrophic	5 M	7-14	18 (11-26)	12 (8-14)	1.9 (1.3-2.8)	4.7 (3.9-5.6)	0.3 (0.1-0.5)	< 0.3
7	Muscular dystrophy, adult type	3 M, 4 F	5-33	18 (9-38)	10 (6-14)	2.6 (1.0-6.0)	4.1 (2.6-6.3)	0.3 (0.2-0.4)	< 0.3
5	Poliomyelitis	4 M, 1 F	6-37	14 (10-17)	11 (8-13)	1.3 (1.0-1.6)	4.5 (3.0-5.2)	0.6 (0.2-0.4)	< 0.3
7	Amyotonia congenita	4 M, 3 F	7-27	9 (7-17)	9 (4-20)	1.2 (0.8-1.7)	4.5 (3.3-6.3)	0.3 (0.1-0.5)	< 0.3
6	Paraplegic	3 M, 3 F	16-32	5 (0-16)	17 (14-22)	1.1 (0.8-1.4)	5.0 (3.4-6.2)	0.7 (0.3-0.8)	< 0.3
6	Quadriplegic	4 M, 2 F	18-28	5 (4-6)	15 (12-24)	1.4 (0.8-1.9)	5.7 (4.9-6.8)	0.5 (0.3-0.8)	< 0.3
4	Polymyositis	1 M, 3 F	31-45	9 (7-10)	8 (4-12)	1.4 (0.7-1.5)	4.2 (2.4-5.3)	0.5 (0.4-0.6)	< 0.3
5	Disuse atrophy	3 M, 2 F	30-65	4 (0-16)	11 (5-17)	0.8 (0.5-1.4)	5.0 (3.7-7.9)	0.7 (0.4-0.9)	< 0.3
2	Charcot-Marie-tooth peroneal atrophy	1 M, 1 F	10-40	4 (0-7)	23 (20-27)	1.2 (1.2)	4.2 (4.2)	0.5 (0.3-0.7)	< 0.3
2	Amyotrophic lateral sclerosis	2 M	43-51	4 (0-7)	16 (15-18)	0.8 (0.5-1.0)	3.7 (2.6-4.8)	0.7 (0.7-0.8)	< 0.3
2	Dermatomyositis	1 M, 1 F	13-40	14 (14)	7 (7)	—	5.0 (4.0-6.6)	0.4 (0.2-0.5)	< 0.3
4	Myotonia dystrophia	4 M	46-57	3 (2-5)	14 (10-21)	1.4 (1.3-1.4)	5.0 (3.5-5.9)	0.6 (0.6)	< 0.3
3	Myasthenia gravis	1 M, 2 F	32-60	0 (0)	20 (19-23)	0.8 (0.6-1.0)	2.8 (1.3-4.0)	0.8 (0.6-0.9)	< 0.3
3	Progressive muscular atrophy	2 M, 1 F	30-71	8 (5-9)	13 (12-15)	1.7 (1.6-1.9)	4.0 (3.0-6.3)	0.4 (0.2-0.5)	< 0.3

urine were analyzed for the guanidinium compounds by the method of Van Pilsum and associates.[16] Blood and urine guanidine and methylguanidine were measured only in the samples from patients with poliomyelitis and muscular dystrophy.

RESULTS

The results are shown in Table I. The normal values are expressed as the average ± the standard deviation; the abnormal values are expressed as the average and their ranges. In all muscle disorders except myasthenia gravis there was a creatinuria usually accompanied by an elevated blood creatine, an elevated urine guanidinoacetic acid, and a decreased urine creatinine. There was no significant trend in blood creatinine levels and no increase in blood guanidinoacetic acid. Blood and urine arginine levels were not reported because of their great variation with no evident trends in any group. No guanidine was found in the blood or urine (above levels of 0.05 mg./100 ml. or 2 mg. per day, respectively) from patients with poliomyelitis or muscular dystrophy. No methylguanidine was found in the blood or urine (above levels of 0.02 mg./100 ml. or 1 mg. per day, respectively) from patients with poliomyelitis or muscular dystrophy.

DISCUSSION

The metabolic role of the guanidinium compounds is outlined in the following manner. Creatine phosphate, an energy store in muscle physiology, is formed by the phosphorylation of creatine in muscle.[17] Creatine is synthesized in the liver by the methylation of guanidinoacetic acid.[18] Guanidinoacetic acid is formed in the kidney from the amino acids arginine and glycine.[19] Guanidine and methylguanidine have no known function in the body and in fact have not been identified as normal body constituents.[16]

A characteristic pattern of guanidinium compound levels was not found for any one type of muscle disorder but rather was found to be similar for all the disorders. If the muscle cells were unable to "utilize" creatine it might be expected to be found in increased amounts in the blood—and such was found to be the case. Perhaps the elevated blood creatine levels retarded the methylation of guanidinoacetic acid to creatine in the liver with the resultant increase of guanidinoacetic acid excreted in the urine. The analytical method for blood guanidinoacetic acid may not have been sensitive enough to measure any increase in blood levels of the compound or its removal from the blood to the urine may have been too fast to produce any increase in the blood.

The decreased creatinine excretion can best be explained with the decreased "utilization" of creatine, presumedly the conversion to creatine phosphate. The spontaneous conversion of creatine and creatine phosphate to creatinine accounts for the entire amount of creatinine excreted in the urine.[20] Borsook and coworkers[20] found that the rate of conversion of creatine phosphate to creatinine is 4 times the rate of conversion of creatine to creatinine. Any decrease in the creatine phosphate content of the body would thus result in a decreased formation of creatinine.

The previous reports that blood guanidinine and methylguanidine levels increase approximately tenfold (from 0.2 to 2.0 mg./100 ml.) in poliomyelitis[14] can best be explained by the nonspecificity of the analytical methods used.

SUMMARY

Blood and urine samples from patients with various muscle disorders were analyzed for their content of the guanidinium compounds with the use of newly developed methods of increased specificity. In all the disorders (except myasthenia gravis) there was a creatinuria usually accompanied by an elevated blood creatine, an elevated urine guanidinoacetic acid, and a decreased urine creatinine. There was no demonstrable change in blood levels of creatinine or guanidinoacetic acid. Blood and urine arginine levels were variable and showed no definite trend. It was found, contrary to the reports of others, that blood levels of guanidine or methylguanidine did not increase in poliomyelitis or muscular dystrophy.

We wish to thank the following staff members from the University of Minnesota Medical School for their aid in obtaining blood and urine samples from patients: H. Bernhisel, Department of Neurology; F. J. Kottke, M. Koseak, M. E. Knapp, S. J. Gault, B. Sandler, W. G. Kubicek, H. M. Sterling, and G. Gullickson of the Department of Physical Medicine and Rehabilitation; K. Absolon, Department of Surgery; L. Peltier, Department of Orthopedics, R. R. Teeter, Department of Psychiatry and Neurology, J. Anderson, R. L. Vernier, J. H. Bornhofen, S. Sanfilippo, R. Tolson, K. F. Swaiman, and E. Hughes, Department of Pediatrics, and R. M. Fusaro, Department of Medicine, Division of Dermatology. We wish to thank: R. W. Koucky and F. Anderson from Fairview Hospital and C. Jarvis from St. Paul Childrens Hospital for blood and urine samples from children; N. E. Quam, Ebenezer Home, for blood and urine samples from the aged; G. Frank, Minneapolis General Hospital, and S. L. Mandel, Medical Arts Building, Minneapolis, for pathologic samples. We wish to thank T. Bruich and the personnel in the North Clinic, the University of Minnesota Hospital, and V. George, Mount Sinai Hospital, D. Heckman, and D. Berman for drawing blood samples. We would like to express our appreciation to the freshman medical and veterinary medical students and to the graduate students and faculty in Physiological Chemistry for normal blood and urine samples.

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PLASMA URIC ACID LEVELS, URIC ACID EXCRETION, AND RESPONSE TO PROBENECID IN TOXEMIA OF PREGNANCY

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RAISED plasma uric acid levels in eclampsia and pre-eclamptic toxemia have been reported by various authors,[1-4] but the mechanism producing this elevation has not been fully clarified. Evidently the uric acid excretion is reduced even when the glomerular filtration rate is normal or only slightly lowered.[3, 5, 6] The excretion of uric acid is governed by filtration through the glomeruli and subsequent tubular reabsorption, and it has, therefore, been assumed that in the presence of normal glomerular filtration in the toxemic state the hyperuricemia is due to increased tubular reabsorption, with or without increased production, of uric acid.[3, 5, 6]

Investigation of the mechanism of increased tubular reabsorption of uric acid in toxemia, and its relation to other physiologic disturbances found in this condition, requires a knowledge of its incidence, of the time of its appearance in relation to other disturbances, and should include an attempt to abolish or modify it by experimental or therapeutic measures.

Since it has been shown that in gout the high plasma uric acid level can be lowered by uricosuric agents such as probenecid,[7-9] it appeared worthwhile to examine the effect of this drug on the renal excretion of uric acid in toxemic women. This question seemed of additional interest because it has been claimed[9] that the effectiveness of this drug in gout depends on the presence of normally functioning kidneys, although other authors have reported that also in gouty subjects with chronic renal disease the drug increases uric acid excretion.[10]

The following is a report of a study of the renal clearance of uric acid in toxemia of pregnancy and of the changes in the uric acid excretion effected by probenecid.*

MATERIAL AND METHODS

The subjects for this study were 15 women suffering from toxemia of pregnancy who presented, in various degrees, the classic signs of this condition: edema, albuminuria, and elevated blood pressure. They had no history of previous or simultaneous renal disease and

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This article is based in part on a dissertation presented by one of the authors (W. J. C.) in December, 1956, in partial fulfillment of the requirements for the degree of Doctor of Medicine of the Hebrew University-Hadassah Medical School.

Received for publication June 25, 1957.

*We are indebted to Teva Ltd., Jerusalem, Israel, for a generous supply of Benuryl [p-(di-n-propylsulfamyl)-benzoic acid].

normal values were obtained, prior and during the study, for blood urea, urea clearance, and creatinine clearance. Except for the albuminuria, there were no other abnormal urinary findings on repeated examinations.

Ten normal young persons, 3 of them pregnant women, were studied as a control group. None of these control subjects had any signs of renal disease.

Both patients and control subjects received a normal ward diet but those with toxemia were moderately restricted in their intake of salt.

Daily creatinine and uric acid clearances were estimated using the total 24-hour urine. Blood samples were drawn in the fasting state. After 3 to 4 days of control study, the subjects were given 0.5 Gm. of probenecid 3 times daily for an additional 5 to 6 days. The clearance tests were continued for some days after the drug was stopped. In certain cases the drug was then administered for a second similar period.

Uric acid levels in the plasma and urine were determined by the method of Folin, as modified by Hepler.[11] Creatinine levels were determined by the method of Hare.[12]

TABLE I. DAILY URINARY OUTPUT, CREATININE CLEARANCE, PLASMA URIC ACID LEVELS, 24-HOUR URIC ACID EXCRETION, RENAL URIC ACID CLEARANCE, AND TUBULAR URIC ACID REABSORPTION IN 10 NORMAL SUBJECTS AS WELL AS IN 15 TOXEMIC WOMEN BEFORE AND AFTER ADMINISTRATION OF PROBENECID

CASES	I. URINE VOLUME (C.CM./24 HR.)		II. CREATININE CLEARANCE (C.CM./MIN.)		III. PLASMA URIC ACID (MG. %)		IV. URINARY URIC ACID EXCRETION (MG./24 HR.)		V. URIC ACID CLEARANCE (C.CM./MIN.)		VI. TUBULAR REABSORPTION OF URIC ACID IN PER CENT OF LOAD	
CONTROLS	A	B	A	B	A	B	A	B	A	B	A	B
1	1,300	1,200	98	95	3,5	3,0	500	527	10,0	12,2	90,0	87,0
2	1,200	1,200	93	98	3,6	3,0	470	600	9,1	13,9	91,2	87,0
3*	1,000	1,100	96	94	3,4	3,0	420	510	8,6	11,8	91,0	87,5
4*	1,200	1,100	108	100	3,6	3,0	470	500	9,0	11,6	91,5	85,0
5	1,300	1,200	103	95	4,7	4,3	650	830	9,6	13,4	91,5	86,0
6	1,200	1,400	105	105	3,7	3,0	500	705	9,4	16,3	92,2	84,5
7	1,200	1,300	100	105	3,7	3,2	420	525	7,9	11,4	92,2	89,9
8	1,300	1,000	110	108	3,8	3,2	610	543	11,1	11,8	90,0	89.0
9	1,400	1,200	104	100	3,4	3,0	480	526	9,8	12,1	92,5	88,0
10*	1,200	1,300	98	105	4,2	3,9	500	800	8,3	14,2	93,0	88,5
Mean	1,210	1,200	101,5	100,5	3,8	3,3	502	606	9,3	12,9	91,5	87,2
S.D.					0,385	0,44	70,2	119,5	0,88	1,485	0,95	1,62
Toxemia												
1	1,000	1,000	99	99	5,9	3,7	300	650	3,5	12,2	96,0	88,0
2	1,050	1,100	98	102	4,9	2,6	350	780	5,0	20,8	95,0	79,8
3	920	950	102	100	5,0	2,4	320	600	4,4	17,4	95,5	82,5
4	850	800	105	99	6,5	2,1	350	720	3,7	23,8	96,5	76,0
5	800	1,000	100	98	5,8	2,7	240	840	2,9	21,6	97,0	78,0
6	1,300	1,300	110	103	6,3	3,0	340	730	3,7	16,8	96,5	83,5
7	1,200	1,200	95	107	4,9	2,9	330	560	4,7	13,4	95,0	87,5
8	1,100	1,250	96	100	7,2	4,0	480	835	4,6	14,5	95,0	75,0
9	1,200	1,300	105	109	7,0	4,5	380	1,200	3,8	18,5	96,0	83,0
10	1,000	1,000	95	90	10,2	5,2	460	1,270	3,1	17,0	96,5	81,5
11	1,100	1,000	100	105	6,8	3,2	320	850	3,3	18,5	96,5	82,5
12	1,300	1,200	105	108	5,9	2,8	380	900	4,5	22,3	96,0	79,0
13	1,500	1,300	108	107	9,8	3,1	290	805	2,1	18,0	98,0	83,0
14	1,400	1,500	96	107	10,2	2,8	340	1,130	2,3	28,0	97,5	73,5
15	1,200	1,300	98	104	6,7	3,2	320	895	3,3	18,4	97,5	81,5
Mean	1,128	1,147	101	102	6,9	3,2	347	851	3,7	18,8	96,3	81,0
S.D.					1,7	0,8	51.3	201,2	0,84	3,96	0,91	4,0

*Pregnant women.

RESULTS

It should be noted in Table I that columns A contain the average results of the 3- to 4-day period prior to the administration of probenecid, whereas columns B contain the figures obtained only during that day of medication in

which the uric acid clearance was maximally elevated. This method of presentation was adopted because the effect of the administration of probenecid on the excretion of uric acid and on the plasma uric acid level developed only gradually. Fig. 1, *A* and *B*, demonstrates this gradual effect, both on a normal and toxemic patient.

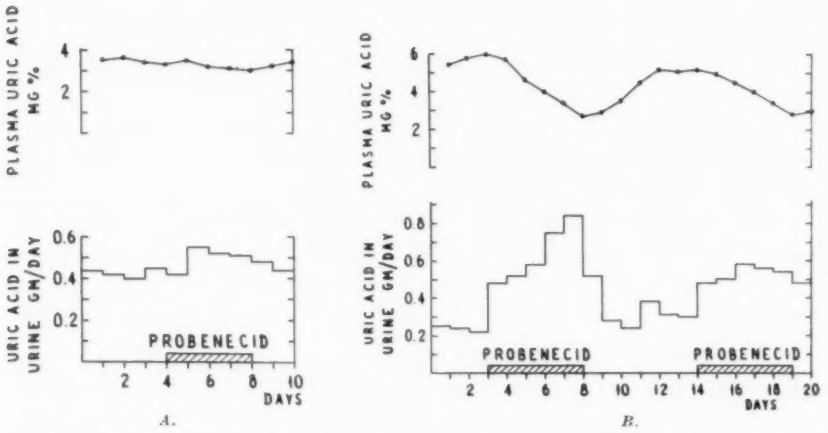

Fig. 1.—*A*, Effect of probenecid on plasma uric acid levels and daily uric acid excretion in a normal control subject. *B*, Effect of probenecid on plasma uric acid levels and daily uric acid excretion in a patient with toxemia of pregnancy.

It can be seen that the 24-hour creatinine clearance and urinary output were approximately the same in both groups.

The average plasma uric acid level in the control subjects was 3.8 ± 0.38 mg. per cent, while the corresponding figures for the toxemia patients were 6.9 ± 1.7 mg. per cent ($t = 5.4$).

The uric acid excretion in the controls was 502 ± 70.2 mg. per day and in the toxemic patients 347 ± 51.3 mg. per day ($t = 5.7$). This difference is even more pronounced when the figures of uric acid clearance are compared due to the factor of the plasma uric acid level which enters into the clearance equation. The figures were 9.3 ± 0.88 c.c. per min. for the normal subjects and 3.7 ± 0.84 c.c./min. for the toxemia patients ($t = 15.5$).

In both groups of subjects the administration of probenecid caused a gradual increase in the daily uric acid excretion, the plasma uric acid level decreasing concomitantly. However, the difference between the two groups in their behavior toward the drug was very pronounced. The daily excretion of uric acid in the group of controls rose from 502 ± 70.2 to 606 ± 119.5 mg. ($t = 2.25$, $p < 0.05$), whereas in the group of toxemic women the rise was from 347 ± 51.3 mg. to 851 ± 201.2 ($t = 9.0$). The uric acid clearance in the control group increased from 9.3 ± 0.88 c.c. per min. to 12.9 ± 1.49 c.c. per min. ($t = 6.8$), in the toxemia group from 3.7 ± 0.84 to 18.8 ± 3.96 c.c. per min. ($t = 14$).

Expressed in terms of tubular reabsorption of uric acid in relation to its filtered load, the figures show a decrease caused by probenecid from 91.5 ± 0.95 per cent to 87.2 ± 1.62 per cent in the control group ($t = 6.8$) and from 96.3 ± 0.91 per cent to 81.0 ± 4.0 per cent in the toxemic patients ($t = 14.2$).

The decrease in the plasma uric acid level caused by the uricosuric action of the drug was not significant in the control group, from 3.8 ± 0.38 to 3.3 ± 0.44 mg. per cent ($t = 1.09$, $p > 0.2$), but was marked in the group of toxemic women, being from 6.9 ± 1.7 to 3.2 ± 0.8 mg. per cent ($t = 7.1$).

It can be seen that the "t" values, expressing the differences between the periods prior to and during the administration of the drug were much higher in the toxemic group of patients than in normals. Further, direct comparison of the results shows that the toxemia patients differed significantly from the controls not only by higher initial plasma uric acid levels and lower uric acid clearance, but also by the much more pronounced effect of probenecid on the tubular reabsorption and ultimate excretion of uric acid.

When the administration of probenecid was stopped, the urinary uric acid excretion and the blood uric acid levels returned to premedication values in both groups within 2 to 3 days.

In 3 of the patients with toxemia the administration of probenecid was resumed after an interval of 8 days. The results during this second period were very similar to those in the first, although somewhat less pronounced. (See Fig. 1, *B*).

DISCUSSION

1. Uric Acid Excretion and Plasma Uric Acid Levels in Toxemia.—The results obtained during the premedication periods accord with previous reports[1-6] that the excretion of uric acid is reduced in toxemia patients, the mechanism responsible being a greater tubular reabsorption of uric acid from the glomerular filtrate. According to our results, which showed the creatinine clearance to be within normal range, a reduction in glomerular filtration rate does not seem to play a part in the reduced uric acid clearance in toxemic patients.

The finding of a significantly elevated plasma uric acid level in toxemic patients, as compared with normals, corresponds to the findings of most other observers. The conclusion drawn by Seitchik[6] from a comparison of the uric acid level in mild toxemia and in normal ante-partum patients that hyperuricemia is not characteristic of mild pre-eclampsia, may in part be explained by the rather high value of 6.0 mg. per cent of plasma urate which was adopted by this author as the upper limit of normal. An analysis of his figures actually reveals a statistically significant difference in the plasma urate level between the two groups.

The mechanism in toxemia of pregnancy producing the elevated plasma uric acid level and the reduced uric acid clearance, without reduction in glomerular filtration rate, is obviously different from that causing hyperuricemia in advanced renal insufficiency. In the latter condition uric acid retention is closely related to the reduction in the glomerular filtration rate, although

the clearance of uric acid is not always reduced to the same extent as other renal functions. There is also probably no similarity to the hyperuricemia of gout, which according to modern views is caused by increased production rather than by diminished excretion uric acid,[13] although increased tubular reabsorption is still held by some authors to be partly responsible for the high plasma uric acid levels found in this condition.[14]

As seen from our figures, in confirmation of the findings of other authors, the deviation from normal in the renal excretion of uric acid in the toxemic state is not connected with any demonstrable changes in other renal functions. Essentially there is an increase rather than a decrease of an isolated tubular function, i.e., the tubular reabsorption of uric acid from the glomerular filtrate. This situation may be compared to the increased tubular reabsorption of salt in many conditions in which the kidneys are not at fault, and in which hormonal influences are at play. It seems possible, therefore, that the tubular reabsorption of uric acid is also governed by an extrarenal mechanism, which appears to be overactive in the toxemic state. Such an assumption finds support in the observations made in this study that in each individual case the renal clearance of uric acid and the plasma uric acid level, although differing from normal to various degrees, showed a remarkable stability during the whole premedication period, and returned almost exactly to their premedication levels after the administration of probenecid was discontinued.

2. Response to Probenecid in Toxemia of Pregnancy.—Increased urinary uric acid excretion and lowering of the plasma uric acid level after administration of probenecid (Benemid) to toxemic patients has also been reported by Hayashi,[15] but no other renal functions were tested in his study and no conclusion drawn as to the mode of action of the drug in this condition.

Our observations are in accord with those of other workers who found that the tubular reabsorption of uric acid is markedly inhibited by this drug and neither changes in urine flow nor in glomerular filtration rate are responsible for the increased uric acid excretion.[8, 16] However, from our figures it appears that there exists a significant quantitative difference in the response to probenecid between normal subjects and toxemic patients. The maximal values for uric acid excretion are much higher in the toxemia group than in the group of normals. That this difference is not due to the initially higher plasma uric acid levels, but to a more pronounced depression of tubular reabsorption of uric acid in the toxemic patients can be seen most clearly in column VI of Table I, in which the figures for tubular reabsorption are expressed as per cent of the filtered load. It must be assumed, therefore, that in the toxemic state there exists an enhanced sensitivity of the renal tubules toward probenecid, which is much higher than under normal conditions. We are unable, however, to state whether the response to probenecid in toxemic patients is also different from that in patients with gout. The relative sensitivity of all these groups—normal subjects, patients with gout, and toxemic women—still remains to be established by direct comparison using identical techniques.

SUMMARY

Women suffering from toxemia of pregnancy were found to have significantly higher plasma uric acid levels and lower urinary uric acid excretion than normal subjects. This was shown to be due to increased tubular reabsorption of uric acid, without signs of other disturbance of renal functions.

Probenecid increased the daily excretion of uric acid in the toxemic patients to a much higher degree than in the normals. In the toxemic state the tubular reabsorptive mechanism for uric acid appears to be more sensitive to probenecid than under normal conditions.

We are indebted to Professors B. Zondek and A. Sadowsky, Department of Gynecology and Obstetrics, for their helpful cooperation in this study.

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EXPLORATION OF THE PHAGOCYTIC FUNCTION OF THE RETICULOENDOTHELIAL SYSTEM WITH HEAT DENATURED HUMAN SERUM ALBUMIN LABELED WITH I^{131} AND APPLICATION TO THE MEASUREMENT OF LIVER BLOOD FLOW, IN NORMAL MAN AND IN SOME PATHOLOGIC CONDITIONS

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WHEN proper colloidal suspensions of particle sizes which do not cross the capillary barrier are injected intravenously, the particles are phagocytized by the reticuloendothelial (R.E.) cells lining the blood vessels and principally by the Kupffer cells of the liver and the reticular cells of the spleen.[1-4] The blood clearance of such suspensions follows an exponential function of the time $C = Co\ 10^{-KT}$. The constant K of this equation varies inversely with the amount of colloid injected, $K \times D = ct.$[3, 4] above a critical dose; in this range, K, which has been called the phagocytic index, is a measurement of the phagocytic activity of the R.E. cells investigated.

Below the critical R.E. dose, the rate of clearance tends to be constant and independent of the amount of colloid injected. In this range the liver absorbs nearly 100 per cent of the injected colloid with a maximum efficiency of clearance, and the colloid clearance test is dependent only upon the blood flow through that organ. Under these experimental conditions, the values obtained are independent of the nature of the injected colloid and do not measure the phagocytic function of the R.E. cells.

The colloid clearance test in the maximum clearance range has been used by various authors to measure liver blood flow with different products (chromium phosphate,[5, 21] colloidal gold,[6, 7] and human serum albumin complex tagged with I^{131} [4, 12]) with comparable results. The measurement of liver blood flow by these techniques gives low values because the maximum efficiency of colloid clearance by the liver is not 100 per cent; although it approaches that value,[4-6] a correction must be made for this factor.

The colloids used in man to measure liver blood flow could not be injected in sufficient amounts to be in the range where $K \times D = ct.$ and, therefore, could not be used to assess the phagocytic activity of the R.E. cells.

Under proper conditions, heat treatment of serum proteins leads to the formation of soluble aggregates[8, 9] which are phagocytized very efficiently by the R.E. cells when injected intravenously.[4, 10, 11] We have prepared and standardized a complex of heat denatured human serum albumin, labeled with

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Received for publication July 5, 1957.
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I^{131}, which, because of its lack of toxicity, its stability in the blood, and absence of antigenicity in the homologous animal species, can be injected in large and small doses to explore both the R.E. cell phagocytic activity and to measure liver blood flow in laboratory animals[4] and in man.[12] In the present study, we present the detailed results of measurements with this colloid of the phagocytic activity of the R.E.S. and liver blood flow in normal man and in patients with some pathologic conditions.

METHODS

Preparation of Albumin Complex (C.A.).—The human serum albumin used was the preparation (supplied by the Centre National de la Transfusion Sanguine) available in a 25 per cent solution for clinical use. This solution contained mendelic acid 0.05 M as a stabilizer and it had been already heated at 60° C. for 10 hours in order to sterilize it. This treatment is not sufficient to modify the protein and render it susceptible to phagocytization by the reticuloendothelial system (R.E.S.).

All procedures were carried out with sterile technique. A dilution containing 1 per cent albumin was prepared with physiologic saline and the pH adjusted to 7. Heating was carried out as previously described.[4]

The solution was then divided in two batches. A portion was iodized with iodine containing I^{131} after albumin complex was isolated,[4] while the other was iodized without radioactive iodine in order to be able to vary independently the dose of labeled albumin complex (C.A.I.) and the amount of radioactivity.

To save one step in the preparation, iodization of the albumin complex without I^{131} was carried out before isolation of the albumin complex from the heated solution. Then, isolation of the nonradioactive iodized albumin complex was carried out by the procedure used to isolate the uniodized C.A. from the heated solution.[4] The iodization was performed as described below for radioactive C.A.I.^{131} except that no I^{131} is used.

Iodization of C.A. with I^{131}.—Solutions and amounts used for 100 mg. of protein: Na I^{131} carrier free, 2 mc. per milliliter 1.33 ml. of a solution of KI_3 containing 123 mg. of iodine and 157 mg. of KI per 100 ml. was employed as well as 1.53 ml. of a carbonate buffer at pH 10 $NaHCO_3$ 0.05 M, and Na_2CO_3 0.15 M.

The solution containing I^{131} is added to the solution of KI_3 and 20 minutes are allowed for the distribution of the radioactive iodine in I_2 and KI.

Meanwhile, the carbonate buffer is added to the solution of C.A. which is cooled to about 4° C. Then, the solution of KI_3 containing I^{131} is added while shaking. The radioactivity of the solution is measured in the proper dilution. After one hour, the solution is dialyzed against normal saline at pH 7.4 until the saline contains only negligible amounts of radioactivity. All procedures are carried out at 4° C. After dialysis, the radioactivity of the solution is measured again to calculate the percentage of iodine attached to the protein.

The amount of iodine which is bound to the C.A. with this technique is well below 1 per cent and usually about 0.5 per cent.

Two solutions of C.A.I. are obtained, one containing I^{131}. The solutions contained usually between 20 and 30 mg./ml. of iodized albumin complex. To ensure sterility, phenol in the concentration of 0.25 per cent was added. Several batches were prepared. Blood clearance rates of these preparations were measured in guinea pigs weighing about 300 grams with the dose of 2 mg./100 Gm. of C.A.I.^{131}. The results were found to be homogenous. The mean phagocytic index K recorded was 0.046 ± 0.007. These values are given as control method of biologic standardization of the C.A.I.^{131} for use in man.

Injection and Dosage of C.A.I.^{131}.—C.A.I.^{131} was injected intravenously in one arm and blood samples drawn from a vein in the other arm at suitable times into heparinized tubes. The radioactivity was measured in the plasma in a well scintillation counter keeping the volume constant.

The two solutions of radioactive and nonradioactive C.A.I. were mixed in convenient proportions, so as to inject the proper dose of C.A.I. and a suitable amount of radioactivity to allow accurate measurements.

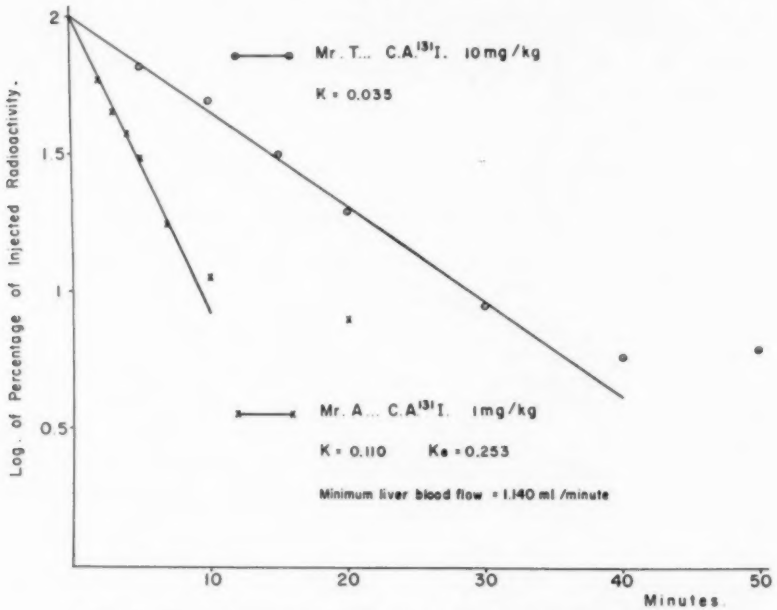

Fig. 1.—Blood clearance of 1 and 10 mg./Kg. of C.A.I.^{131} in 2 normal men.

In the first experiments, up to 56 μc of I^{131} were injected; then, with the improvement of the techniques of measurement, the dose could be reduced to 10 μc of I^{131}.

The dose of 10 mg./Kg. of C.A.I.^{131} (about 20 ml.), well within the range where $K \times D = ct.$ in man, was injected slowly in 3 minutes to explore the phagocytic activity of the R.E.S. in 7 normal and in 12 pathologic cases. The rate of clearance of C.A.I.^{131} was measured.

The phagocytic index K was calculated from equation 1:

$$\frac{\text{Log } C_1 - \text{Log } C_2}{T_2 - T_1} = K \text{ (decimal logarithms).}$$

There remained a small but variable amount (10 per cent) of radioactivity in the blood at the end of the experiment (Fig. 1) due probably to the rapid breakdown of the phagocytized C.A.I.^{131} and release of radioactivity in the circulation[4]; the phagocytic index was calculated from the straight portion of the curve.

The dose of 1 mg./Kg. of C.A.I.^{131} which is below the range where $K \times D = ct.$ in man was injected to measure liver blood flow in 5 normal subjects and in 6 patients with liver cirrhosis.

The rate of clearance of C.A.I.^{131} was measured. The specific rate constant Ke was calculated from Naperian logarithm and equation 1. As above, the portion of the curve was considered where the concentration is an exponential function of the time (Fig. 1).

Plasma volume was calculated in all cases by extrapolating to 0 time the clearance curve of C.A.I.^{131}. Blood volume was calculated by correcting for the hematocrit.

Calculation of liver blood flow was made by multiplying the value of the blood volume by Ke for each subject. No correction was made for the efficiency of liver clearance which was not measured in man. The values obtained should, therefore, be qualified as "minimum liver blood flow."

In the hours and days that followed the injections, measurement of gamma radioactivity over the liver, spleen, thyroid, and other organs were performed with the scintillation counter.

The amount of radioactivity excreted in the urine was also measured for each patient until the excretion became negligible.

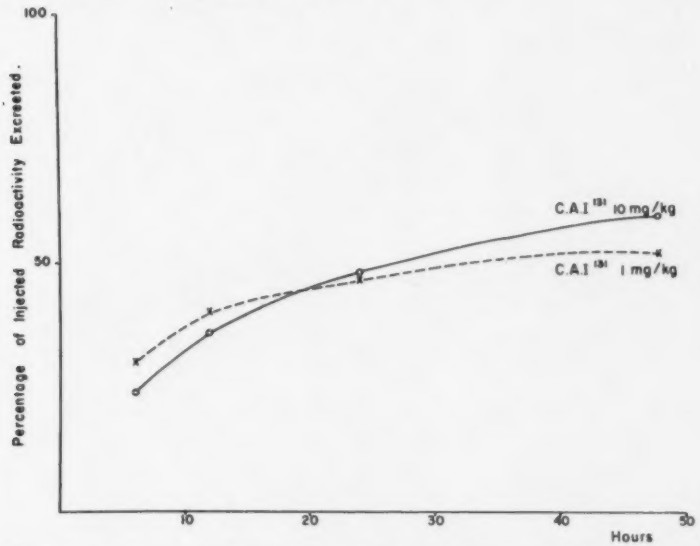

Fig. 2.—Rate of urinary excretion of injected radioactivity after 1 or 10 mg./Kg. of C.A.I.^{131} in normal adults. Mean values of several determinations.

RESULTS

In the 30 examinations reported, the injection of C.A.I.^{131} was perfectly well tolerated without signs or symptoms irrespective of the dose; no signs of shock, pyrexia, or other abnormal effects were recorded in spite of the fact that 3 patients were injected twice with C.A.I.^{131} at 1 or 2 week intervals. However, in one case of chronic leukemia in an aged female patient who had received several blood transfusions with frequent reactions, a transient shock-like reaction was observed without lasting effects.

The dose of I^{131} injected was generally well below the dose usually used to explore thyroid activity. Moreover, about 50 per cent of the radioactivity is eliminated in the urine in 24 hours (Fig. 2).

Examinations performed after the blood clearance of the C.A.I.^{131} on the various organs with the scintillation counter showed that the injected radioactivity can be localized chiefly in the liver and spleen. No significant accumulation of I^{131} is then found in the thyroid. Later, the radioactivity of

the liver and spleen decreases to negligible values after 6 days, while the thyroid shows some uptake of I^{131}. The thyroid uptake can be limited by administration of Lugol's solution previous to the test.

In Fig. 1, we present 2 typical examinations performed on 2 normal subjects. One patient received 10 mg. of C.A.I.^{131} per kilogram of body weight to explore the phagocytic activity of the R.E.S., another patient was injected with 1 mg. per kilogram to measure liver blood flow. The phagocytic index K and the specific rate constant Ke are calculated as described above, neglecting the residual blood radioactivity which may be due to a release of radioactivity from the R.E. cells.[4]

TABLE I. PHAGOCYTIC ACTIVITY OF THE R.E.S. MEASURED BY THE PHAGOCYTIC INDEX K FOR THE DOSE OF 10 MG. OF C.A.I.^{131} PER KILOGRAM IN 7 NORMAL ADULTS

NAME	SEX	AGE	WEIGHT (KG.)	DOSE IN MICRO-CURIES I^{131}	PHA-GOCYTIC INDEX K	BLOOD VOLUME		HEMATO-CRIT (PER CENT)
						ML.	ML./KG.	
Lak.	M.	24	58	56	0.045	4,900	84	49
Teb.	M.	53	64	56	0.035	4,220	66	46
Lar.	M.	28	61	52	0.039	5,300	87	45
Che.	M.	20	70	52	0.034	4,150	60	47
Our.	M.	30	56	19	0.042	4,500	80	50
Ger.	F.	54	61	32	0.022	4,400	72	45
Ous.	M.	29	57	32	0.048	5,100	89	46
Mean Values			61		0.038 ± 0.008	4,650	77	

In Table I, the data concerning the R.E.S. phagocytic activity towards C.A.I.^{131} in normal subjects are reported. The examinations were performed on 6 men and 1 woman. The values of K in the 6 men investigated varied very little, range from 0.034 to 0.048, irrespective of age. The one woman studied gave a low value 0.022. The blood volume calculated from the same data gave an average of 77 ml. of blood per kilogram of body weight. In Table II, we present the results of exploration of R.E.S. phagocytic function in some pathologic conditions.

The values of K observed in 2 cases (5 and 6) of Hodgkin's disease undergoing treatment with radiotherapy and cortisone, in one case (3) of chronic myeloid leukemia, and in one case of chronic lymphatic leukemia (4) are within the normal range recorded in the controls.

In 2 cases (1 and 2) of acute leukemia, the values of K are slightly below the standard deviation of the normal subjects. It should be noted that Subject 1 is a woman and that the only normal woman investigated gave a similar value of K.

Subject 7, also a woman, with an untreated reticulum cell sarcoma gave an elevated phagocytic index 0.050, outside of the normal standard deviation.

In 4 cases of liver cirrhosis, at various stages of evolution, we found the phagocytic index K to be normal in patients without ascites and decreased significantly in 2 patients (11 and 12) with ascites.

In Table III, we present the values of minimum liver blood flow in 5 normal men and in 6 cases of liver cirrhosis of varied severity together with the blood volume and hematocrit values calculated from the same data.

TABLE II. PHAGOCYTIC ACTIVITY OF THE R.E.S. MEASURED BY THE PHAGOCYTIC INDEX K FOR THE DOSE OF 10 MG. OF C.A.I.^{131} PER KILOGRAM IN 7 NORMAL ADULTS

NO.	NAME	DIAGNOSIS	SEX	AGE	WEIGHT (KG.)	DOSE IN MICROCURIES I^{131}	PHAGOCYTIC INDEX K	BLOOD VOLUME		HEMATOCRIT (PER CENT)
								ML.	ML./KG.	
1	Noy.	Acute leukemia	F.	66	56.5	7	0.024	5,300	94	28
2	Cau.	Acute leukemia	M.	55	65	11	0.029	5,400	83	18
3	Nec.	Chronic myeloid leukemia	M.	57	77	10	0.037	5,450	71	46
4	Ger.	Chronic lymphatic leukemia	M.	66	52	32	0.036	4,100	79	28
5	Leg.	Hodgkin's disease	M.	31	58	10	0.040	4,120	71	41
6	Jou.	Hodgkin's disease	M.	22	56	17	0.037	5,000	89	34
7	Mon.	Reticulum cell sarcoma	F.	49	52	21	0.050	6,000	115	25
8	Thi.	Congenital hemolytic anemia	F.	31	44	22	0.040	4,300	98	33
9	Min.	Banti's disease	F.	49	52	22	0.038	3,300	63	32
10	Aut.	Liver cirrhosis without ascites	M.	50	60	32	0.030	4,400	73	32
11	Bel.	Liver cirrhosis with ascites	M.	54	64.5	10	0.025	6,000	93	35
12	Juc.	Liver cirrhosis with icterus	M.	49	75	13	0.020	4,600	61	30

TABLE III. MEASUREMENT OF "MINIMUM LIVER BLOOD FLOW" FROM BLOOD CLEARANCE OF 1 MG. PER KILOGRAM OF C.A.I.^{131} IN 5 NORMAL ADULTS AND IN 6 CASES OF LIVER CIRRHOSIS

NAME	SEX	AGE	WEIGHT (KG.)	DIAGNOSIS	DOSE IN MICROCURIES I^{131}	KE NAP. LOG.	BLOOD VOLUME		HEMATOCRIT (PER CENT)	MINIMUM LIVER BLOOD FLOW (ML./MIN.)
							ML.	ML./KG.		
Abb.	M.	20	57,5	Normal	17	0.314	4,400	77	48	1,380
Arr.	M.	33	66	Normal	7	0.253	4,500	68	49	1,140
Hau.	M.	24	54	Normal	24	0.156	5,600	104	51	880
Mag.	M.	47	61	Normal	20	0.193	5,100	84	46	1,000
Mut.	M.	30	57	Normal	26	0.216	4,100	72	46	890
			Mean Values			0.226	4,750	81	--	1.060 ± 180
Aut.	M.	50	61	Liver cirrhosis without ascites	17	0.240	3,800	62	35	910
Ave.	F.	59	52	Liver cirrhosis without ascites	18	0.178	3,170	61	39	570
Pag.	M.	50	93	Liver cirrhosis without ascites	10	0.129	5,600	60	30	720
Lem.	M.	48	60	Liver cirrhosis with ascites	46	0.123	3,850	64	30	475
Mar.	F.	66	86	Liver cirrhosis with ascites	30	0.099	3,800	44	40	375
Bel.	M.	54	63	Liver cirrhosis with ascites	10	0.092	5,800	92	44	530

The average value of minimum liver blood flow observed with our technique was 1.060 ± 180 ml. per minute, the mean specific rate constant Ke was 0.226. The average blood volume was 81 ml./Kg.

In liver cirrhosis without ascites, the liver blood flow decreases as both Ke and the blood volume are lowered in 2 out of 3 cases. In the 3 cases of liver cirrhosis with ascites, there is a considerable decrease of liver blood flow, the constant Ke is found to be less than half the normal value.

DISCUSSION

The lack of toxicity and the stability of the heated albumin allow the injection of a sufficient amount of colloid to explore the phagocytic function of the R.E.S. in man. The dose of 10 mg./Kg. of C.A.I.^{131} gives an average value of K of 0.038. The value of K × D in man for this substance is 0.38, where the dose is expressed per kilogram of body weight. The theoretical critical R.E. dose of C.A.I.^{131} will be 0.38/0.098 = 3.9 mg./Kg. in man. This shows that 10 mg./Kg. is well in the K × D = ct. range and 1 mg./Kg. below the critical R. E. dose. 10 mg. per Kg. of C.A.I.^{131} is an appropriate dose to explore the phagocytic activity of the R.E. cells and 1 mg./Kg. is suitable to measure liver blood flow.

The values of the phagocytic index reported for the standard preparation of C.A.I.^{131} depend, of course, on the nature of the preparation and the dose injected. The preparations have been standardized by both physical methods and biologic assay in laboratory animals, and care must be taken to verify each batch in the animal to ensure that the results are comparable. The data reported concern a limited number of pathologic cases. The only cases studied that deviated from the normal standard deviation significantly were one patient with reticulum cell sarcoma, who showed an increased phagocytic activity of the R.E.S., and 2 patients with liver cirrhosis with ascites, where the value of K was decreased. This last finding, however, does not indicate a lowered function of the R.E. cells themselves in liver cirrhosis, but it is rather the result of decreased blood flow through the liver in these patients.

The actual measurement of liver blood flow was made on one of these two patients, and the value obtained was considerably decreased. After phagocytosis of C.A.I.^{131} by the R.E. cells of the liver and spleen, the radioactivity disappears very rapidly from these organs and the I^{131} is excreted by the kidney for the greatest part in 48 hours. Further studies should be made to investigate the breakdown products containing I^{131} in the blood and in the urine.

The colloid clearance test both for the dose of 10 mg./Kg. or 1 mg./Kg. of C.A.I.^{131} can be used to calculate blood volume. The average value obtained from our 12 normal subjects was 78 ml. of blood/Kg. of body weight with a mean uncorrected hematocrit value of 47 per cent.

The results were in agreement with values reported by various authors[6, 21] using the Evans blue dye or tagged red cells to measure blood volume. Nachman and associates[14] found 81 ml. of blood per kilogram in 38 normal patients; Fauvert and co-workers[15, 16] reported 76 ml./Kg. of blood. Stoll[17] reported 70.7 ml./Kg. in men and 73 ml./Kg. in women.

Several authors working with tagged red cells found somewhat lower values.[18, 19] It is generally agreed that the techniques involving tagged red cells give lower values than the dye method of measuring blood volume.[14, 15, 18, 19]

It was, therefore, felt that the respective values of blood volume obtained with our technique were reliable and could be used to calculate the liver blood flow in each patient.

The data concerning "minimum liver blood flow" in normal subjects is known to be less than actual blood flow, since the hepatic clearance of C.A.I.^{131} is not 100 per cent and the factor of correction was not measured in man. In laboratory animals (rats, guinea pigs, and rabbits), the hepatic clearance is fairly constant, around 80 per cent.[4]

The values of minimum liver blood flow in normal man reported by other authors using the colloid clearance technique[6, 7, 21] or Bromsulphalein[20, 22, 23] are higher than those reported here. This is due to greater values of Ke found by the investigators using the colloid clearance test.[6, 7, 21] It is possible that higher values of Ke can be calculated from our data if correction is made for the residual radioactivity (Fig. 1) by subtracting it from the experimental values.

The standard preparation of C.A.I.^{131} used was designed primarily to explore the phagocytic activity of the R.E.S. and was also applied to the measure of liver circulation. In order to come closer to actual values, the method of preparation can be changed to yield an albumin complex consisting of larger aggregates, more suitable for the measurement of liver blood flow.

In liver cirrhosis, the amount of blood flowing through the liver is generally decreased. The extent of the diminution of liver blood flow, which has been observed also by other authors,[7, 24] varies with the severity of the disease; patients with ascites show lower liver blood flow than patients without ascites.

SUMMARY

A method is presented to explore the phagocytic function of the reticuloendothelial system in man, by measuring the speed of clearance from the blood of an appropriate dose of heat denatured and aggregated human serum albumin labeled with I^{131}.

The average value of the phagocytic index K in 7 normal subjects was 0.038 to 0.008.

The phagocytic function of the R.E.S. was investigated in a few cases of acute leukemia, chronic leukemia, Hodgkin's disease, reticulum cell sarcoma, and liver cirrhosis.

The same technique is used to measure hepatic circulation by decreasing the dose of C.A.I.^{131} injected to the maximum liver clearance range. The value of liver blood flow obtained in 5 normal subjects and in 6 patients with liver cirrhosis are reported.

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SPHEROCYTIC HEMOLYTIC ANEMIA ASSOCIATED WITH ACUTE PNEUMOCOCCAL INFECTION IN RABBITS

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A LARGE body of information has been collected concerning the anemia of chronic infection. In contrast, few data are available concerning the anemia which frequently accompanies acute bacterial infections in young human beings. Because antibiotics and chemotherapeutic agents have shortened the course of most bacterial infections it is difficult to investigate the pathogenesis of the anemia of acute infection in human subjects. Therefore, an analogous experimental model was sought.

An acute inflamatory process can be produced in rabbits by the intradermal injection of living pneumococci.[1] Jacox and Feldmahn[2] noted that rabbits infected in this manner became anemic. The following study was undertaken to define the nature of the anemia thus produced experimentally in rabbits.

MATERIALS AND METHODS

Twenty-one male albino rabbits weighing from 1,670 to 3,790 grams were used for the experiments. Hematologic studies and measurements of weight and rectal temperature were made on two or three occasions prior to infection of the animals and daily thereafter. Blood samples were obtained from the marginal ear vein. The skin about the vein was shaved and prepared with xylene. Any trace of the latter was carefully removed prior to incision of the vessel. A small artery clamp was placed over the vein at the base of the ear, and the vessel near the ear tip was incised with a sharp blade. The first few drops of blood were discarded. Approximately 0.5 ml. of blood was then allowed to drip into a paraffin-coated watch glass. The daily blood loss seldom exceeded 1 ml.

Duplicate determinations of packed red cell volume were made in capillary tubes.[3] Reticulocytes were stained with new methylene blue,[4] and their percentage was estimated after enumeration of 1,000 erythrocytes. Smears of blood taken directly from the vein were prepared by the cover-glass method.[5] The erythrocyte osmotic fragility was measured at pH 7.0 using the method described by Young and associates[6]; the 1.5 ml. of blood required for this procedure were collected in a 10 × 75 mm. test tube containing a small amount of powdered heparin.† The range (two standard deviations from mean) of concentrations of sodium chloride causing 2, 5, 25, 50, and 75 per cent hemolysis was calculated from the results of studies of fresh blood from 18 normal rabbits. Measurements of erythrocyte osmotic fragility were made prior to and at appropriate times during the experimental infection.

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This work was supported by a Postdoctoral Research Fellowship of the National Institutes of Health, U. S. Public Health Service.

Received for publication July 9, 1957.

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The technique of preparation and injection of the suspension of microorganisms has been described by Jacox and Feldmahn.[2] When a drop of the suspension was viewed microscopically, 5 to 15 microorganisms were visible per oil immersion field. One-tenth milliliter of this suspension of living Type I pneumococci was injected intradermally into 15 of the rabbits. Henceforth, these rabbits will be referred to as the "infected" group of animals. One-tenth milliliter of an isotonic solution of sodium chloride was administered in a similar manner to 6 other rabbits. These animals were subjected to the same experimental procedures as the former group and served as the controls. Henceforth, they will be referred to as "noninfected" or "control" animals.

RESULTS

Twenty-four hours after inoculation of the pneumococci the animals were febrile and exhibited edema and redness about the site of injection. Bacteremia has been shown to occur 24 to 48 hours after the intradermal injection of Type I pneumococci.[1, 2] Penicillin in doses varying between 500 and 18,000 units was administered to 10 of the 15 infected animals to prolong their illness by preventing early death. In two instances (Rabbits 5 and 16) the administration of the antibiotic resulted in premature recovery from the infection.

The animals injected with saline remained in good health and exhibited no reaction at the site of injecton. Penicillin in amounts comparable to those received by the infected rabbits was administered to 5 of the 6 control animals.

Three of the 15 rabbits infected with Type I pneumococci died within 3 days of the onset of the infection. No qualitative or quantitative changes were apparent in the erythrocytes of these 3 animals. The remaining 12 rabbits of this group all exhibited decreases of their packed red cell volumes; the maximum diminution occurred 3 to 8 days (Average, 5.2 days) after inoculation of the microorganisms (see Table I, *A*). The approximate volume of blood withdrawn between the day of onset of the infection and the day on which the lowest packed red cell volume was noted varied between 3 and 11 ml. (Average, 5.6 ml.) per animal.

The control rabbits exhibited no consistent change of their hematocrit measurements. For purposes of comparing these animals with the infected rabbits, Table I, *B*, records the changes which were observed in the noninfected group on the sixth day after injection of the saline solution. On this day the control rabbits' cumulative blood losses varied between 6 and 7 ml. (Average, 6.2 ml.) per animal.

With one exception the control animals gained weight during the 6 days following the injection of the saline solution. Four of these control animals exhibited decreases of their hematocrit values; the greatest change was observed in Rabbit 23 whose packed red cell volume decreased 6 per cent. The fall of the hematocrit in this rabbit was accompanied by a 15 per cent increase of body weight.

In contrast, all of the infected animals lost from 5 to 23 per cent of their original body weights. With these weight losses there were concomitant losses of skin turgor. The packed red cell volumes of 7 of the infected rabbits decreased 8 to 12 volumes per cent. Of these 7 animals, 4 had cumulative blood losses less than that of control animal 23.

TABLE I. SUMMARY OF OBSERVATIONS

A. Observations on Rabbits Subjected to Pneumococcal Infection

ANIMAL NO.	WEIGHT PRIOR TO INFECTION (GM.)	DAY AFTER INFECTION WHEN LOWEST HCT. NOTED (DAY)	CHANGE OF PRCV BETWEEN DAY OF INFECTION AND DAY OF LOWEST HCT. (PER CENT PACKED R.B.C.)	PER CENT WEIGHT CHANGE BETWEEN DAY OF INFECTION AND DAY OF LOWEST HCT. (PER CENT)	APPROXIMATE TOTAL BLOOD LOSS BETWEEN DAY OF INFECTION AND DAY OF LOWEST HCT. (ML.)	RETICULOCYTES* PRIOR TO INFECTION (PER CENT)	RETICULOCYTES THIRD DAY AFTER INFECTION (PER CENT)	RETICULOCYTES DAY OF LOWEST HCT. (PER CENT)	SPHEROCYTOSIS	INCREASE† OF ERYTHROCYTE OSMOTIC FRAGILITY DURING INFECTION	COURSE OF INFECTION
1	2,295	3	-10.5	-8	3	7.0	2.5	2.5	+	-	Died fourth day
2	2,666	3	-3.5	-13	3	4.3	1.0	1.0	-	-	Died fourth day
3‡	2,739	3	-2.0	-17	4	6.0	2.1	0.5	-	-	Died sixth day
4‡	2,452	8	-12.0	-18	5	2.2	1.2	8.0	+	-	Survived
5‡	2,704	3	-6.0	-5	3	1.9	2.8	2.8	0	-	Recovered prematurely
6	2,649	-	-	-	-	2.2	-	-	-	-	Died second day
7‡	3,086	4	-12.0	-10	4	3.6	1.8	2.2	+(?)	-	Died sixth day
8‡	2,561	6	-10.5	-5	8	3.6	0.5	2.5	+	+	Sacrificed eighth day
11‡	2,166	8	-9.5	-22	11	3.2	2.9	5.7	+	+	Survived
12	2,140	-	-	-	-	4.1	-	-	-	-	Died third day
13‡	3,324	6	-8.0	-14	7	2.3	1.8	5.0	+	+	Survived
16‡	3,607	7	-1.0	-10	7	2.2	0.5	2.2	+(?)	0	Recovered prematurely
17‡	3,353	-	-	-	-	2.3	-	-	-	-	Died third day
20	2,223	7	-5.5	-10	7	3.0	2.3	1.3	0	0	Survived
22‡	1,673	3	-12.0	-8	5	0.8	1.8	1.3	0	0	Survived

TABLE I—CONT'D

B. Observations on Control Rabbits

ANIMAL NO.	WEIGHT PRIOR TO INJECTION OF SALINE (GM.)	DAY AFTER INJECTION WHEN OBSERVATIONS RECORDED (DAY)	CHANGE OF PRCV BETWEEN DAY OF INJECTION AND DAY 6 (PER CENT PACKED R.B.C.)	PER CENT WEIGHT CHANGE BETWEEN DAY OF INJECTION AND DAY 6 (PER CENT)	APPROXIMATE TOTAL BLOOD LOSS BETWEEN DAY OF INJECTION AND DAY 6 (ML.)	RETICULOCYTES PRIOR TO INJECTION (PER CENT)	RETICULOCYTES THIRD DAY AFTER INJECTION (PER CENT)	RETICULOCYTES SIXTH DAY AFTER INJECTION (PER CENT)	SPHEROCYTOSIS	INCREASE† OF ERYTHROCYTE OSMOTIC FRAGILITY AFTER INJECTION
14‡	2,372	6	−4.5	+4	7	6.0	4.8	12.0	0	0
15‡	2,686	6	+2.0	+1	6	2.3	2.6	3.7	0	0
18‡	3,790	6	+1.5	-2	6	2.7	1.2	1.0	0	0
19‡	3,285	6	−4.0	+2	6	1.9	2.2	2.2	0	0
21	1,860	6	−3.0	+15	6	1.8	1.8	2.3	0	0
23‡	1,891	6	−6.0	+15	6	0.8	2.8	3.0	0	0

HCT. = Hematocrit; PRCV = Packed Red Cell Volume.
*Normal values = 2.1 ± 0.8 per cent.
†An increase of erythrocyte osmotic fragility beyond the normal range (two standard deviations of mean).
‡Animals which received penicillin.

In one of the control animals there was a reticulocytosis of 12 per cent 6 days after the saline injection; in this instance, however, the proportion of reticulocytes was elevated prior to the injection of the salt solution. Reticulocytosis was apparent in 3 rabbits 6 to 8 days after the onset of the pneumococcal infection. No animal of either group exhibited a proportion of reticulocytes below the normal range. In most of the rabbits infected by pneumococci decreases of the proportion of reticulocytes were apparent during the first few days of the illness; this trend was not as striking in the control group.

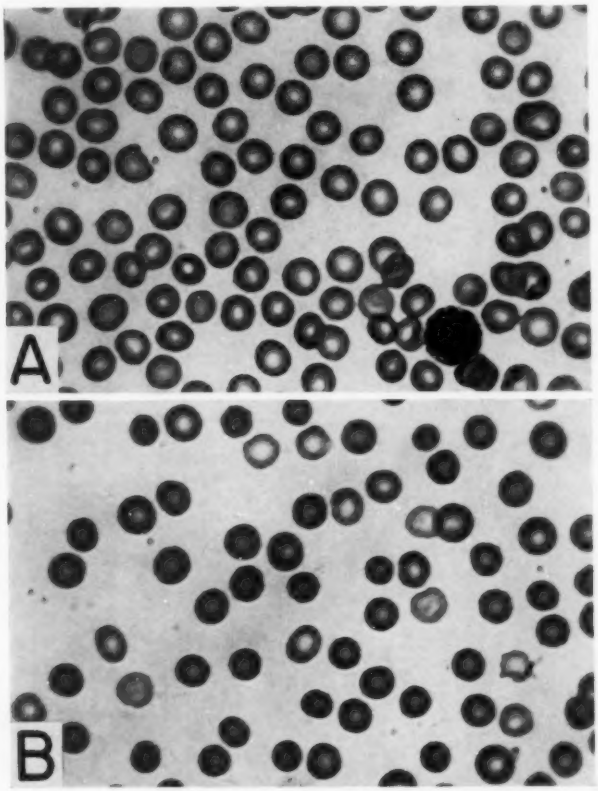

Fig. 1.—*A*, Photomicrograph of blood smear of Rabbit 4 prior to infection with Type I pneumococci. *B*, Photomicrograph of blood smear of Rabbit 4 eight days after inoculation with Type I pneumococci. Note the spherocytosis. (Magnification ×970; reduced ⅔.)

Spherocytosis became evident in the blood smears of 5 of the rabbits during the course of the pneumococcal infection (see Fig. 1). This erythrocyte abnormality was not observed in any of the control animals. Measurements of erythrocyte osmotic fragility were made prior to and during the course of the

illnesses of 6 of the infected animals. Increases of erythrocyte osmotic fragility were noted during the infection in 3 of these 6 rabbits (see Fig. 2). Similar studies of the 6 control animals disclosed no significant changes of erythrocyte osmotic fragility. As Rabbits 11 and 13 recovered from their pneumococcal infections the results of their erythrocyte osmotic fragility tests became normal. The return of erythrocyte osmotic fragility to normal was completed within 10 days in Rabbit 11 and within 5 days in Rabbit 13.

No gross evidence of bleeding into the tissues was apparent in Rabbits 1, 2, and 8 on which autopsies were performed.

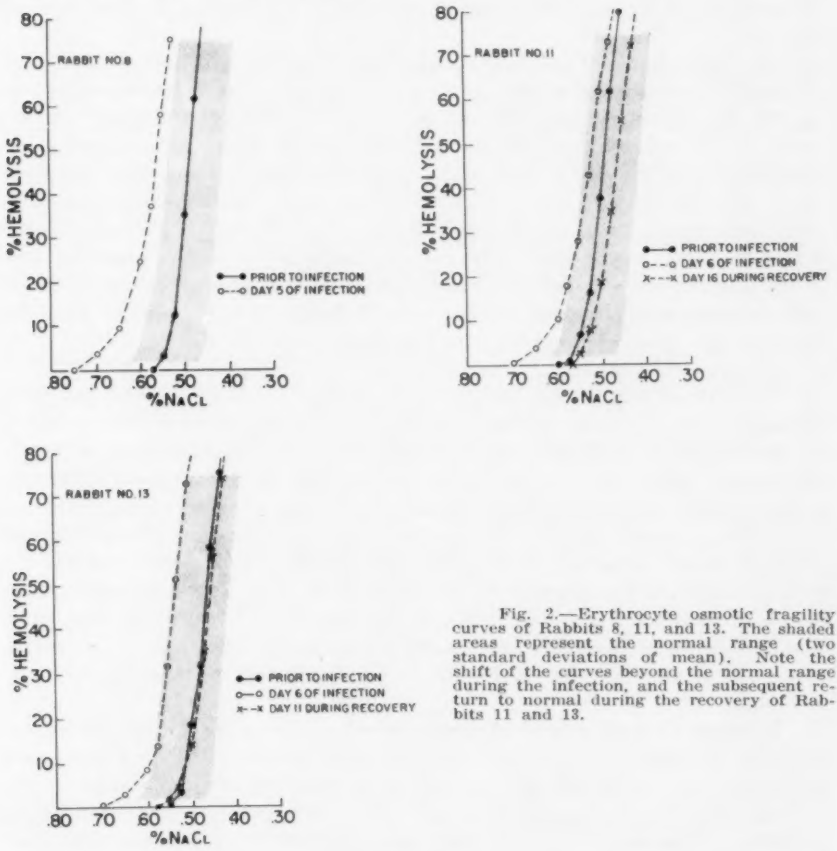

Fig. 2.—Erythrocyte osmotic fragility curves of Rabbits 8, 11, and 13. The shaded areas represent the normal range (two standard deviations of mean). Note the shift of the curves beyond the normal range during the infection, and the subsequent return to normal during the recovery of Rabbits 11 and 13.

DISCUSSION

From the data presented it is apparent that several of the rabbits infected by the Type I pneumococci exhibited greater decreases of their packed red cell volumes than did any rabbit of the control group. These changes were evident despite dehydration which presumably resulted in hemoconcentration.

Therefore, it seems probable that the decrement of total red cell volume in many of the infected rabbits was greater than the decrement of total red cell volume in any of the noninfected animals. The amounts of blood withdrawn were small and were approximately equal in the two groups of rabbits; it is unlikely that this factor contributed greatly to the anemia.

The decrease of the proportion of reticulocytes in the first few days of the bacterial infection may be indicative of some suppression of erythropoiesis. This is not surprising in view of the results of studies of the anemia of chronic infection.[7] However, there was no apparent cessation of erythrocyte production which might contribute significantly to a diminution of total red cell volume.

The occurrence of spherocytosis and the rapidity with which the anemia developed in the rabbits subjected to the pneumococcal infection can be regarded as indirect evidence for the existence of a hemolytic process. The development of spherocytosis during the illness and its disappearance during convalescence provide firm evidence of red cell damage accompanying the infection. The nearly symmetrical shifts in the curves of osmotic hemolysis indicate that major proportions of the erythrocytes were affected. The rapidity with which the erythrocyte osmotic fragility subsequently returned to normal suggests that the red cell damage was reversible. The mechanism by which the spherocytosis was produced has yet to be defined. It is conceivable that this experimental model is analogous to the anemia associated with certain acute infections of childhood.

Extracts of certain strains of *Vibrio cholerae*[8, 9] and most strains of pneumococci[10] will in vitro render erythrocytes panagglutinable (the so-called T-transformation of Thomsen and Friedenreich). T-Transformation of erythrocytes has been produced in vivo in guinea pigs by infection with Type 19 pneumococci[10] and by the intravenous administration of an ezyme extracted from that microorganism.[11] The intravenous administration of concentrated T-transforming enzyme to guinea pigs has produced a hemolytic anemia the severity of which was thought to be related to the high levels of T-agglutinins in the animals prior to the injection of the enzyme.[11] Because the concentration of T-agglutinins in the rabbit is low,[8] it seems unlikely that this phenomenon could account for the spherocytic hemolytic anemia described in the present report.

Extracts of some strains of pneumococci contain a heat labile hemolysin which in its reduced form is actively hemolytic in vitro.[12, 13] The intravenous administration of the purified hemolysin to mice resulted in sudden death without evidence of hemoglobinemia.[14] However, the injection of a crude unheated pneumococcal extract into mice resulted in anemia the nature of which was not elucidated. Whether these observations are pertinent to the hemolytic anemia described in the present report remains to be determined.

SUMMARY

A spherocytic hemolytic anemia accompanying a severe acute pneumococcal infection of rabbits has been demonstrated. The procedure described provides another method for producing spherocytosis in vivo.

I wish to express my gratitude to Dr. Gerald Miller for his advice and encouragement and to Miss Norma Trabold for her technical assistance.

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CARDIAC OUTPUT MEASURED BY THE INJECTION METHOD WITH THE USE OF RADIOACTIVE MATERIAL AND CONTINUOUS RECORDING

II. Comparison With the Fick Method

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THE injection method for determining the cardiac output has been used for many years.[1-3] Most investigators have used T-1824 dye as the injected material. The concentration of the injected dye may be determined on arterial blood samples collected every 1 to 2 seconds as done by Hamilton,[3] or by means of an oximeter with continuous recording of the dye concentration as employed by Friedlich[4] and Warner.[5]

In 1951, MacIntyre[6] reported the use of radioactive iodinated human serum albumin and a continuous arterial sampling technique. In 1956, we[7] presented the results of circulation model experiments using a modification of their technique employing a well-type scintillation counter and the pip interval recording method. We now present a comparison of the cardiac output values as determined by the pip interval recording method with the results of the Fick method for cardiac output and report the results of repeat cardiac output determinations using the pip interval method in the same subject.

METHOD

The apparatus, method, and a statistical analysis have been presented elsewhere.[7] In brief the method was as follows. Arterial blood was drawn from the 18 gauge Cournand needle in the subject through disposable polyvinyl tubing which was held in rigid geometry within a well crystal by a Lucite or aluminum mold (Fig. 1). The concentration of the radioactivity was recorded by using the energy of the output of the register circuit of a scaler to produce small pips on a recording oscillograph. The counting rate was determined for each one second interval, graphed, and the cardiac output calculated according to the conventional method. A standard dilution of the injected material made with carrier plasma was counted in the same geometry as the continuous sample.

For this study a saline solution of RISA* was prepared for injection. In those subjects in which the injection was made into the antecubital vein, a calibrated syringe was used to inject approximately 75 μc in exactly 3 ml. followed by a rapid flush. In those subjects in which the injection was made into the catheter, approximately 40 μc in exactly 2 ml. was injected into the main pulmonary artery (M.P.A.) by a precision injection pipette[8] with a 10 ml. saline rinse. Although the standard deviation of the delivered

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This investigation was supported by Grant No. H-2195 from the National Institutes of Health, Public Health Service, and by a grant from the Los Angeles County Heart Association.

Received for publication July 17, 1957.

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volume of the injection pipette was ±1.0 per cent as compared with that of ±0.2 per cent for the calibrated syringe, the former was used in those cases in which the injection was made into the catheter because of greater ease of injection. No other changes were made in the method as previously reported. A more flexible substitute for the motor-driven syringe was found in a peristaltic action pump* which regulated the sampling rate to a constant flow, for this application 50 ml./min. This flow rate changes the volume of the sample within the well about twice per second.

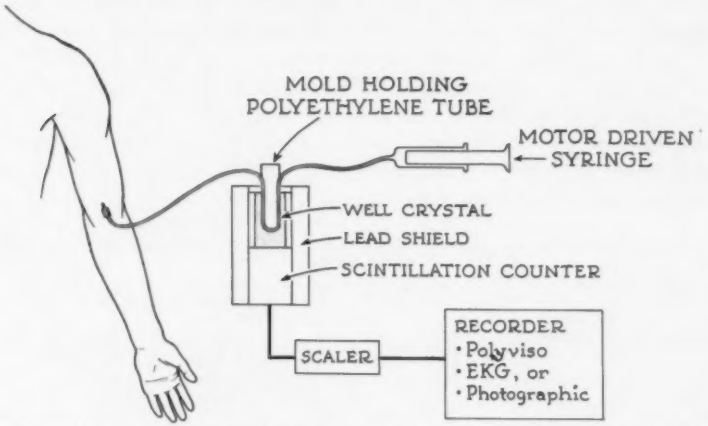

Fig. 1.—Apparatus arrangement for determining cardiac output. (From Crane et al. J. LAB. & CLIN. MED. 47: 802, 1956.)

The method was compared with the Fick method of cardiac output at catheterization. Nine patients were studied in which the RISA was injected into an antecubital vein and 19 were studied in which the injection was made into the M.P.A. In these patients the following plan was used. A 3-minute expired air sample was collected. During the middle minute the arterial and mixed venous blood samples were obtained. As soon as feasible after the Fick samples were obtained, usually within 15 minutes, the RISA cardiac output determination was made. The gas sample was analyzed by the Scholander method.[9] The blood hemoglobin oxygen content was determined by the Van Slyke[10] manometric method. The cardiac output was calculated by means of the Fick principle.

Duplicate cardiac output determinations were made on 22 patients with various disease entities to determine the reproducibility of the method. In this portion of the study the following course was followed. After the first cardiac output determination, 15 minutes was allowed for complete mixing of the RISA. A sample was then withdrawn for determination of whole blood volume, and the second cardiac output was run immediately. A second whole blood volume determination was made allowing 15 minutes for mixing. A correction was made for the background material in the blood and the amount of blood withdrawn for the first test.

In the comparison with the Fick method for cardiac output all patients were included in the study except those in which there were known technical errors and those in which there was a circulatory shunt as demonstrated by either the Fick analysis or the RISA curve. None of the patients in the repeat determination group had known shunts.

RESULTS

Table I shows the comparison between the Fick output and the RISA outputs. The per cent difference between the two methods ranges from –15.3

*Model No. 5-8950, American Instrument Company, Silver Springs, Md.

to +26.8 per cent. The upper portion presents the comparison of the two methods in 9 patients in which the injection was made by calibrated syringe into an antecubital vein. The average per cent difference between the Fick output and the RISA output was −1.9 per cent (S.D. ±9.3). Also shown is a comparison of the two methods in 17 patients in which the injection was made into the M.P.A. The average per cent difference between the Fick output and the RISA output was +1.8 per cent (S.D. ±10.3). By Fisher's test there was no significant difference between the Fick and RISA output values whether the injection was made into the cubital vein ($P > 0.5$) or the M.P.A. ($P > 0.7$). Also there was no significant difference ($P > 0.3$) between the mean per cent differences in the cubital vein and M.P.A. groups. The ratios of the central volumes and the whole blood volumes at the two injection sites are given, and it is noted that the average ratio is 0.50 and 0.26 when injected into the antecubital vein and M.P.A., respectively.

TABLE I. COMPARISON OF THE INJECTION METHOD USING PIP INTERVAL RECORDING WITH THE FICK METHOD

PATIENT	FICK OUTPUT (L./MIN.)	RISA OUTPUT (L./MIN.)	PER CENT DIFF.	CENTRAL VOLUME (L.)	WHOLE B.V. (L.)	C.V. W.B. VOL.
RISA Injected Into Antecubital Vein						
O. B.	3.51	3.83	9.1	2.18	3.90	0.56
A. R.	4.22	3.75	−11.1	1.69	3.75	0.45
S. L.	4.10	3.75	− 8.5	2.37	5.18	0.46
J. D.	2.83	2.98	5.3	1.99	4.21	0.47
B. C.	3.63	3.24	−10.7	1.50	2.97	0.51
O. D.	5.05	5.20	3.0	3.29	6.78	0.49
M. B.	3.26	3.46	6.1	1.73	3.76	0.46
D. E.	3.39	2.87	−15.3	3.25	5.56	0.58
R. L.	3.99	4.19	5.0	1.41	2.95	0.48
	Mean		−1.9			0.50
	S.D.		±9.3			±0.04
RISA Injected Into Main Pulmonary Artery						
E. M.	3.95	4.45	12.7	1.06	5.21	0.20
T. B.	7.66	9.71	26.8	1.61	5.78	0.28
H. F.	4.47	4.51	0.9	1.26	4.44	0.28
J. W.	3.05	3.22	5.6	0.98	4.65	0.21
W. A.	3.03	3.32	9.6	1.69	5.33	0.32
L. J.	4.47	4.89	9.4	2.32	6.06	0.38
E. G.	4.40	4.47	1.6	1.19	3.91	0.30
M. V.	3.50	3.19	− 8.9	1.28	4.67	0.27
K. T.	5.83	5.42	− 7.0	1.26	5.00	0.25
G. M.	3.80	3.23	−15.0	1.13	4.18	0.27
V. H.	3.42	3.01	−12.0	1.03	4.36	0.24
A. F.	4.80	5.16	7.5	1.17	5.44	0.22
G. M.	8.19	7.48	− 8.7	1.67	5.46	0.31
C. H.	3.84	3.64	− 5.2	0.48	4.46	0.11
H. P.	6.07	6.10	0.5	1.38	4.67	0.30
J. B.	4.57	5.05	10.5	1.06	4.56	0.23
D. D.	4.35	4.45	2.3	0.91	4.50	0.20
	Mean		+ 1.8			0.26
	S.D.		±10.3			±0.06

Table II presents a comparison of the repeat determinations of cardiac output and blood volume in 22 patients. The per cent difference between the first and second cardiac outputs ranged from −11.0 to +14.2 with an average difference of +0.7 per cent (S.D. ±7.1). The average difference between the

TABLE II. RESULTS OF REPEATED CARDIAC OUTPUT DETERMINATIONS ON THE SAME SUBJECT

PATIENT	B.S.A. ($M.^2$)	DIAGNOSIS	CARDIAC OUTPUT (L./MIN.)			MEAN CIRC. TIME (SEC.)			CENTRAL VOLUME (L.)			WHOLE BLOOD VOLUME (L.)		
			FIRST	SECOND	% DIFF.	FIRST	SECOND	% DIFF.	FIRST	SECOND	% DIFF.	FIRST	SECOND	% DIFF.
J. M.	1.44	Congestive failure	3.61	3.48	− 3.6	36.6	37.0	1.1	2.20	2.15	− 2.3	5.80	6.09	5.0
M. B.	1.62	Congestive failure	3.16	2.93	− 7.3	39.4	37.6	− 4.6	2.08	1.83	−12.0	4.30	4.24	− 1.4
H. K.	1.71	Congestive failure	4.16	4.38	5.3	23.7	21.8	− 8.0	1.65	1.59	− 3.6	4.04	3.79	− 6.2
M. A.	1.42	Congestive failure	4.22	4.74	12.3	22.5	18.7	−16.9	1.44	1.34	− 6.9	3.74	3.51	− 6.1
E. B.	1.63	Congestive failure	4.35	4.24	− 2.5	35.7	35.6	− 0.3	2.59	2.52	− 2.7	6.38	7.32	14.7
L. B.	2.06	Congestive failure	4.00	4.54	13.5	25.0	27.3	9.2	1.67	2.06	23.4	5.50	5.04	− 8.4
L. L.	1.61	Congestive failure	4.62	4.65	0.6	25.9	25.7	− 0.8	1.99	1.99	0.0	5.22	5.33	2.1
O. H.	2.03	Congestive failure	2.73	2.67	− 2.2	43.8	48.8	11.4	1.99	2.17	9.0	5.49	5.57	1.5
F. L.	1.53	Asthma	5.90	6.09	3.2	18.4	17.9	− 2.7	1.81	1.77	− 2.2	4.45	4.43	− 0.4
J. P.	1.69	Emphysema	5.58	5.76	3.2	26.8	21.8	−18.7	2.49	2.09	−16.1	5.04	5.08	0.8
E. L.	1.61	Emphysema	4.89	4.43	− 9.4	22.0	23.4	6.4	1.79	1.73	− 3.4	3.88	4.19	8.0
O. S.	1.57	Congestive failure	7.28	7.48	2.7	37.0	37.3	0.8	4.49	4.47	− 0.4	7.18	6.96	− 3.1
R. G.	2.22	Obesity	6.14	7.01	14.2	38.0	39.6	4.2	3.89	4.63	19.0	12.29	11.24	− 8.5
O. S.	1.57	Congestive failure	2.69	2.80	4.1	40.0	39.0	− 2.5	2.18	2.24	2.8	4.84	5.57	15.1
F. G.	2.56	Obesity	8.18	8.22	0.5	36.8	39.8	8.2	5.02	5.45	8.6	11.82	11.15	− 5.7
B. Y.	1.92	Emphysema	4.41	4.72	7.0	24.5	25.9	5.7	1.80	2.04	13.3	5.70	5.96	4.6
D. M.	1.77	Emphysema	7.85	7.73	− 1.5	13.8	14.5	5.1	1.81	1.86	2.8	5.53	5.58	0.9
F. E.	1.51	Asthma	5.90	5.25	−11.0	12.3	11.5	− 6.5	1.21	1.00	−17.4	3.54	3.60	1.7
K. J.	2.18	Obesity	6.23	5.57	−10.6	25.3	31.5	24.5	2.63	2.92	11.0	9.05	9.51	5.1
F. G.	2.38	Obesity	9.18	9.48	3.3	15.3	14.5	− 5.2	2.34	2.28	− 2.6	10.27	9.87	− 3.9
J. R.	1.93	Asthma	4.94	4.52	− 8.5	26.8	28.7	7.1	2.21	2.15	− 2.7	6.08	5.78	− 4.9
M. M.	1.58	Congestive failure	2.78	2.84	2.2	35.7	30.9	−13.4	1.66	1.46	−12.0	4.10	3.88	− 5.4
Average per cent difference					+ 0.7			+ 0.2			+ 0.2			+ 0.3
Standard deviation					± 7.1			± 9.6			±10.0			± 6.5

first and second mean circulation times was +0.2 per cent (S.D. ±9.0). The average difference between the first and second central volumes was +0.3 per cent (S.D. ±10.2). The average difference between the first and second whole blood volumes was +0.3 per cent (S.D. ±6.5). The whole blood volumes were noticeably increased in the obesity group.

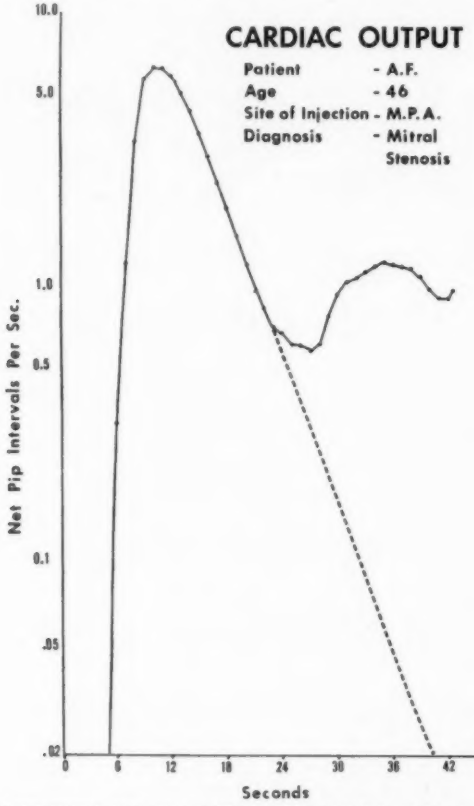

Fig. 2.—A semilogarithmic plot of a patient without left-to-right or right-to-left intracardiac shunt.

In addition to the foregoing data we observed, as have others,[4, 11, 12] that certain shunts can be detected from the configuration of the output curve. The existence only of left-to-right shunts, whether intracardiac or between major vessels, can be shown; whereas right-to-left shunts can be localized by proper selection of the site of injection. Fig. 2 is a normal RISA output curve. Fig. 3 shows a typical RISA curve in a patient with a left-to-right shunt, in this instance due to a patent ductus arteriosus. Note the rather early departure from the exponential decrease of RISA concentration due to early recirculation of a portion of the injected material through the shunt.

Fig. 4 shows two RISA curves in a young patient with an interventricular septal defect. The curve obtained when the injection was made into the M.P.A. has a normal upslope and a downslope which reflects early recirculation compatible with a left-to-right shunt. The RISA curve obtained when the material was injected into the right ventricle shows an early appearance of the material with a distorted upslope having a smaller and then a larger

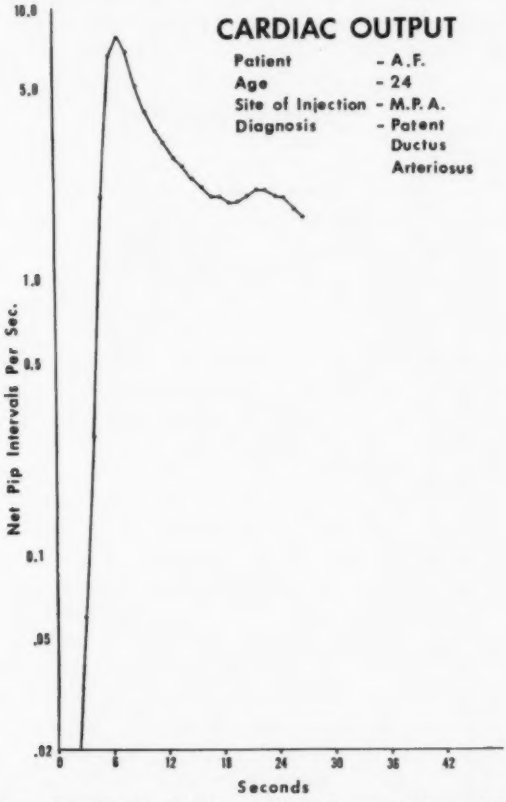

Fig. 3.—A semilogarithmic plot of a patient demonstrating a left-to-right shunt.

peak followed by a downslope similar to the previous injection. We attribute the early appearance of the first peak to a portion of the RISA which traversed the intracardiac shunt and appeared sooner at the peripheral artery than the RISA from the main bolus which traversed the lung.

The curves obtained in congestive failure with low cardiac output have a prolonged appearance time, build-up time, and time for the formation of the downslope resulting in a longer mean circulation time. If the time scale is compressed, the shape of the curve appears identical with a normal tracing.

In a group of 37 patients in Grade III or IV congestive failure (average cardiac index 1.66 L./min./M² [S.D. ±0.44]) in which the injection was made in the antecubital vein the average appearance time from the time of injection was 15.1 sec. (S.D. ±7.6), the peak concentration time was 33.5 sec. (S.D. ±12.3), and the mean circulation time (M.C.T.) was 43.5 sec. (S.D. ±16.4) with a prolonged time base. In 10 normal cardiac outputs of the same age group in which the injection was made similarly the appearance time was 6.8 sec. (S.D. ±2.4), the peak concentration time was 16.8 sec. (S.D. ±4.1), and the mean circulation time was 20.3 sec. (S.D. ±4.9).

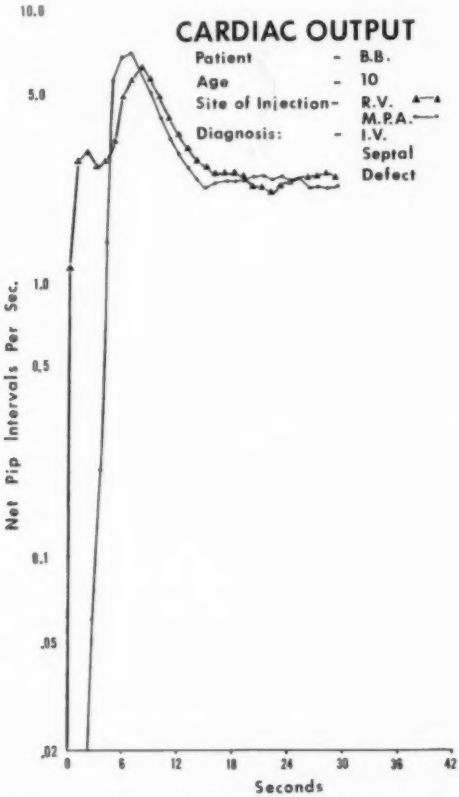

Fig. 4.—A semilogarithmic plot showing both right-to-left and left-to-right intracardiac shunts.

We are currently using a new approach to study the timing and amount of influence of the recirculating indicator upon the initial downslope of the arterial concentration curve in various pathologic states including congestive failure. Preliminary findings indicate that in the absence of a circulatory shunt only an insignificant amount of the recirculating indicator can be shown

to alter the initial downslope before obvious recirculation is demonstrated. This has been true even in congestive failure with an M.C.T. as long as 72 seconds.

DISCUSSION

T-1824 and other dyes have been used extensively in the determination of cardiac output. The dyes present certain handicaps in that there is difficulty in the measurement of their concentration in the presence of free hemoglobin or lipemia. The cyclical variations of arterial oxygen-hemoglobin due to breathing alter the oximetric recordings.[13] With serial determinations the dyes also cause a transient discoloration of the tissues unless one is used which is rapidly excreted.[11]

The use of radioactive isotopes has disadvantages in that certain facilities must be available and the isotope material must of course be periodically replenished. RISA presents other problems in that it tends to stick to glassware and may give erroneous results unless it is handled properly.[14-16] If carrier albumin is used in the preparation of the standard and the volume of injection is near 2 ml. or more, this does not present a problem.[15] In spite of these factors we feel that the advantages of using this material far outweigh its disadvantages.

In order to simplify the determination of cardiac output and to avoid the time lag inherent in the counting-rate computers and ratemeter-recorders we have modified the recording technique from that presented by MacIntyre et al.[5]

Our method allows for a greater range of counting rate without loss in reading accuracy than that afforded by recording ratemeters. If the recorded pips are kept as small as 1 to 2 mm. in height, counting rates as high as 30 pip intervals per second can be recorded at a paper speed of 25 mm. per second. The possibility of the recording going off scale is obviated. Furthermore, repeat determinations with most equipment are hampered because of the limited range of zero suppression of the recording milliameter.

With a ratemeter setting of 0.5 second response time the ratemeter curve is sufficiently erratic to make its reading difficult. With a response time of two seconds the curve is smooth, but rapid changes in counting rates are not detectable. For a direct comparison of the two recording methods the counting rate from a single well-crystal-scaler was channelled simultaneously to the pip interval and ratemeter-Esterline-Angus recording systems for cardiac output and central volume measurements. With the response time setting of 0.5 second the cardiac outputs and central volumes as calculated from the ratemeter recording did not differ significantly from those calculated by the pip interval method. In 18 determinations with a ratemeter response time of two seconds the cardiac outputs averaged 8.8 per cent (S.D. ±9.7) lower by the ratemeter than by the pip interval method and the central volume averaged 13.0 per cent (S.D. ±25.4) greater than by the pip interval method. We feel that the difference is due mainly to the time lag in the ratemeter-recorder.

The use of a continuous sample obviates the laborious collection and analysis of multiple samples. For best results the sampling rate should be

regulated by some means. Free flow from the sampling needle* is imperative. The pump merely maintains the sampling at the desired constant rate. A constant sampling rate will give a more accurate mean circulation time and prevent distortion of the build-up and decay portions of the concentration curve and is very important if the shape of the curve is to be relied upon for the evaluation of shunts. In addition, the fluid within the counting geometry should flow at a sufficient rate to allow a complete change about twice a second. A further advantage of the continuous sampling is seen on the determination of cardiac output in patients who have a low flow-rate with a prolonged appearance time and prolonged time to form the upslope, downslope, and recirculation portion of the curve. In some instances of low cardiac output it may be necessary to sample as long as two minutes to include the first portion of the recirculation curve.

Some attempts have been made by other investigators to measure the cardiac output by external counting over the heart or large vessels. This may give reasonable values for comparative purposes, but the changing vascular geometry and increasing background contribution limits its accuracy. Insertion of an arterial needle presents no particular problem. The use of disposable polyvinyl tubing allows the standard dilution to be counted in the same geometry as the continuous sample.

We have previously reported the accuracy of the method as studied on a laboratory circulation model. The present study shows that this method compares quite favorably with the Fick method for cardiac output. Other comparisons of the Fick method with the injection method have been made. Hamilton[17] and Werko[18] found no systematic error in the injection method when compared with the Fick. The errors in the calculation of the cardiac output using the Fick method are greater than those of the injection method because of the inherent errors in blood gas analysis. The over-all error in the Fick method has been stated not to exceed 15 per cent.[17] The Fick method determines cardiac output over a longer period of time than does the injection method and some of the differences between the two methods may possibly be explained by a cyclic change in cardiac output associated with respiration. However, one would expect the same cyclical change to occur when comparing repeat determinations in the same patient. However, there is no noticeable increased difference between the first and second cardiac outputs which could be attributed to this source. When the injection method as we have used it is repeated on the same patient, the range of per cent difference and the standard deviation of the average per cent difference is noticeably less than when compared with the Fick method. Pritchard and associates[6, 19] have reported that the RISA method for determining cardiac output gave results in dogs which averaged 9 per cent higher with reference to rotameter values (including a 6 per cent correction for coronary flow) and values which averaged 5 per cent higher than the Fick method in patients at cardiac catheterization. They attributed this to a consistent error. We have not found a consistent error in using RISA by our method.

*An 18 gauge needle is large enough for sampling up to 75 ml./min.

A few reports, especially those of Wood and associates,[5, 11, 12] have appeared discussing the application of the injection method for detecting intracardiac shunts. These agree that the configuration of the injection curve can be of help in the detection of shunts within the heart and between major vessels. At the present time this would seem to be the easiest method for localizing right-to-left shunts.

For the localization of a right-to-left shunt the injection should be made in the right auricle or vena cava, the right ventricle, and the main pulmonary artery. Then a curve showing early appearance of the material at a peripheral artery with a double peak would diagnose the shunt and the location of the injection would determine the site of the shunt. The existence of two right-to-left shunts such as shunting between the auricles as well as the ventricles can be demonstrated, but they cannot be separated one from the other by this method.

A left-to-right shunt can be detected also, but cannot be localized with the present techniques. A left-to-right shunt whether due to an atrial septal defect, a ventricular septal defect, a patent ductus arteriosus, or a pulmonary vein emptying into the right heart results in a distortion of the downslope of the curve due to the appearance of a portion of the injected material sooner than the normal recirculation peak. In some defects the amount of the recirculation may be so great that a recognizable downslope does not appear. We agree with Wood[11] in that we have not found a "shunt curve" at catheterization in patients without shunts, and all patients with shunts had curves indicating the same. One patient (L. J.) in whom a shunt pattern was detected by the injection method at catheterization was not verified by the blood gas studies. A repeat catheterization at a later time demonstrated a patent ductus arteriosus.

The curves that we obtained in congestive failure with low cardiac output differed from the normal primarily as a function of time. This change from normal was a prolongation of the appearance time, the build-up time, the formation of the downslope, and thus the prolongation of the mean circulation time. If such a curve is plotted with a compressed time scale it is seen that the shape of the curve is practically identical with that of a normal tracing. The pattern of a left-to-right shunt might be mistaken for a curve of a patient in congestive failure. However, in our experience the shunt curve has normal appearance time and the recirculation peak as seen in congestive failure does not appear. In some instances obviously it would be impossible to diagnose a shunt in the presence of congestive heart failure.

SUMMARY

The pip interval method for determining cardiac output by a continuous sampling and recording method was compared with the Fick method for cardiac output. This report shows that the average difference between the Fick output and the RISA output was −1.9 per cent (S.D. ±9.3) in a group of 9 patients in which the injection was made into an antecubital vein, the average difference between the Fick output and RISA output was 1.8 per cent (S.D. ±10.3) in a group of 17 patients in which the injection was made into

the main pulmonary artery. Consecutive determinations of cardiac output in the same subject by the authors' method in 22 patients shows an average per cent difference between the first and second cardiac output of +0.7 per cent (S.D. ±7.1).

In addition, characteristic left-to-right and right-to-left shunt patterns are presented and discussed.

We wish to acknowledge the assistance of Stanley Weiss, Dr. Liselotte Weinert, and Dorothy Teeple, R.N., with this study.

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THE DISAPPEARANCE RATE OF GLUTAMIC OXALACETIC TRANSAMINASE FROM THE CIRCULATION AND ITS DISTRIBUTION IN THE BODY'S FLUID COMPARTMENTS AND SECRETIONS

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THE release of glutamic oxalacetic transaminase (GOT) from damaged tissues and the significance of serum GOT levels as an index of cell death have been well documented.[1, 2] Little is known of the disappearance rate of GOT from the blood stream or of its distribution in the body's fluid compartments. A knowledge of these parameters is necessary for the understanding of the quantitative and temporal relationship of serum GOT to the extent and activity of tissue necrosis. Following experimental myocardial infarction, the observed increase in serum GOT was far smaller[3] than anticipated from the measured loss of GOT from the infarcted myocardium[4] and the dilution of this quantity of GOT in the plasma volume. This suggests either a rapid removal of GOT from the blood stream or a diluting medium considerably greater than the plasma volume.

In the present study the disappearance rate of GOT from the blood stream was measured following intravenous injection of the enzyme. The distribution of the enzyme in various body fluids was determined by serial measurements of the GOT activity in lymph, spinal fluid, bile, and urine. In addition the distribution and excretion of the enzyme were studied following experimental myocardial infarction, carbon tetrachloride induced hepatocellular necrosis, and in nephrectomized animals.

METHODS

Mongrel dogs were used in all experiments. All animals were fed Purina Dog Chow and water. The enzyme was injected intravenously in the form of crude autogenous or homologous GOT extracts. The autogenous extract was prepared from the animal's excised biceps muscle and was injected after the animal had recovered from the muscle excision. Homologous GOT extracts were prepared from dog liver, heart, and skeletal muscle. The tissues were minced with saline in a Waring blendor; the debris was removed by centrifugation at 3,000 r.p.m.; and the supernatant fluid was passed through a Seitz one micron filter. This filtrate was employed as the GOT extract for intravenous administration.

Myocardial infarction was produced by tightening the free ends of a silk ligature placed loosely around the anterior descending coronary artery one week prior to the experiment. Hepatocellular necrosis was produced by oral administration of 6 ml. of carbon tetrachloride per kilogram of body weight.

In 5 dogs, lymph samples were collected from a cannulated tributary of the thoracic duct. No evidence of interference with lymph flow through the thoracic duct was noted.

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Received for publication July 18, 1957.

Spinal fluid was obtained by cisternal tap. Only samples completely free of blood were used. Bile samples were obtained through external bile fistulae in 5 dogs. The fistulae were established under pentothal anesthesia by inserting a polyethylene catheter into the distally ligated common duct after cholecystectomy. The bile was collected with sterile precautions in a rubber bag bandaged to the abdomen of the dog.

Bilateral nephrectomy was performed on 4 dogs under Nembutal anesthesia. Three of these dogs were given an intravenous injection of GOT extract. The serum GOT was followed until the death of the animals which occurred from 3 to 5 days after nephrectomy. Sham operated animals served as controls.

All GOT measurements were done in a Beckman spectrophotometer according to the method of Steinberg, Baldwin, and Ostrow.[5] Suitable dilutions of the samples in phosphate buffer were made when the GOT activity exceeded 200 units/ml./minute and routinely in bile because of its high optical density. All GOT concentrations were expressed in units per millimeter per minute and corrected to 25° C. In order to test for a possible dissociation of the coenzyme, an aliquot of the bile samples was incubated with varying concentrations of pyridoxal-5-phosphate for one hour and the GOT activity measured.

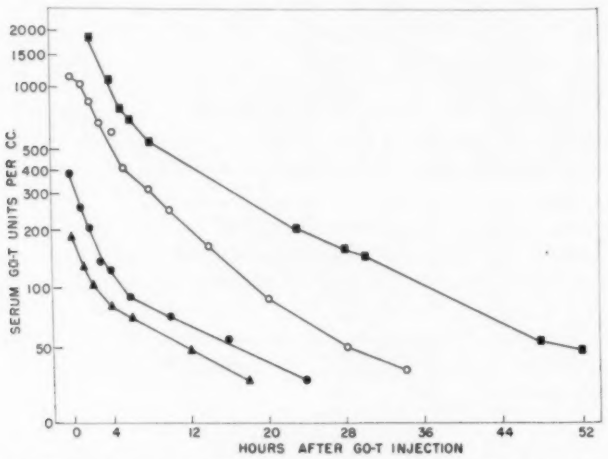

Fig. 1.—Disappearance of GOT from the blood stream after intravenous injection of GOT containing tissue extract (●——●, autogenous muscle extract; ■——■, homologous muscle; ▲——▲, homologous heart; ○——○, homologous liver).

RESULTS

1. *Serum GOT Following Intravenous Injection of GOT.*—Ten minutes after intravenous injection of GOT extract the serum GOT values were compatible with a dilution of the injected quantities in the plasma volumes. Within 6 to 8 hours after injection the serum GOT dropped rapidly to one-fourth of the immediate postinjection level (Fig. 1). Thereafter, a much slower disappearance rate was found and serum GOT returned to a normal range within 18 to 60 hours depending on the amount of GOT injected. The disappearance curves of autogenous and homologous GOT extracts were similar as were disappearance curves of extracts prepared from liver, skeletal muscle, and heart. No measurable amounts of the enzyme were found in the urine of these animals.

2. *GOT Concentration in Thoracic Duct Lymph.*—Since the two distinct slopes of the disappearance curve of GOT from the blood stream indicated at least two reactions, a possible diffusion of GOT into the interstitial fluid was investigated by simultaneous measurements of the GOT activity in lymph and blood. Following intravenous injection of GOT extract, an inverse relationship between the serum and lymph GOT activity was found. Fig. 2 is a representative example of one of five experiments which gave almost identical curves. Equilibration was reached within 6 hours and marked the end of the rapid disappearance period of GOT from the blood. The equilibration level was about one-fourth of the immediate postinjection serum GOT level.

Fig. 3 represents simultaneous measurements of thoracic duct lymph GOT and serum GOT following experimental myocardial infarction. A similar relationship between serum GOT and lymph GOT was seen following carbon tetrachloride intoxication.

3. *Serum GOT Disappearance in Bilaterally Nephrectomized Dogs.*—Since no measureable amounts of GOT were found in the urine after intravenous injection of GOT or following experimental myocardial infarction, the disappearance rate of intravenously injected GOT was studied in bilaterally nephrectomized dogs (Fig. 4) to explore the possibility of an inactivation of GOT in kidneys. No significant differences in the disappearance curves were found when these were compared with a sham operated control (Fig. 4). No significant elevation in serum GOT was seen in one animal not injected with GOT extract during a 4-day observation period following bilateral nephrectomy.

4. *GOT in Spinal Fluid During Elevation of Serum GOT.*—No changes in the spinal fluid GOT concentrations were found during CCl_4 induced hepatocellular necrosis although the GOT serum level exceeded 5,000 units/ml./min. Likewise no elevations in spinal fluid GOT were seen in any experiment after intravenous injection of GOT extracts.

TABLE I. EXCRETION OF GOT IN THE BILE OF TWO DOGS WITH EXTERNAL BILE FISTULA BEFORE AND AFTER INTRAVENOUS INJECTION OF GOT EXTRACT

TIME HOURS	SERUM GOT CONCENTRATION		BILE							
			ML. COLLECTED		GOT CONCENTRATION		TOTAL GOT EXCRETION		24-HR. EXCRETION	
	EXP. 1	EXP. 2	EXP. 1	EXP. 2	EXP. 1	EXP. 2	EXP. 1	EXP. 2	EXP. 1	EXP. 2
-24	External bile fistula established									
-24 to 18	16	14	22	16	30	15	660	240		
-18 to 0	149	175	60	98	10	14	600	1,372	1,260	1,612
0	Intravenous injection of GOT concentrate (approx. 1 million units GOT)									
0 to 2	1,296 997	1,700 1,200	6	8	16	18	96	144		
2 to 4	638	830	7	4	30	35	210	140		
4 to 6	385	610	10	3	14	15	140	45		
6 to 24	95	246	80	42	20	17	1,600	714	2,046	1,043
24 to 48	59	158	100	74	19	14	1,900	1,036	1,900	1,036
48 to 72	19	18	67	60	21	6	1,197	960	1,197	960

Gall bladder bile in Exp. 1: 6 ml., GOT concentration, 198 units/ml.
Gall bladder bile in Exp. 2: 15 ml., GOT concentration, 247 units/ml.

5. *Biliary Excretion of GOT.*—In Table I the serum GOT, bile GOT, and volume of biliary drainage from two dogs with external bile fistula are recorded before and after intravenous injection of GOT extract. Throughout

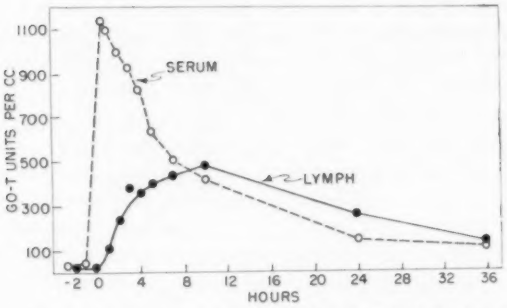

Fig. 2.—GOT concentration in serum and thoracic duct lymph following intravenous injection of GOT.

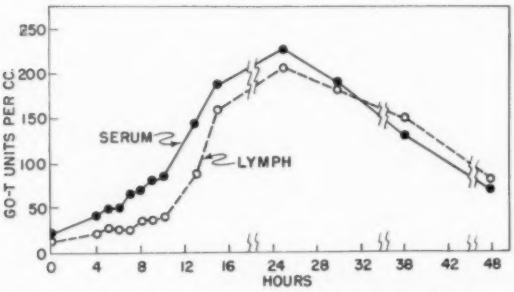

Fig. 3.—Serum and thoracic duct lymph GOT concentration following myocardial infarct.

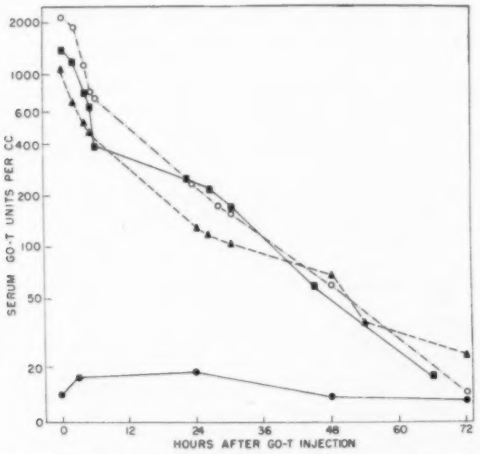

Fig. 4.—Serum GOT concentration in bilaterally nephrectomized dogs (▲- - -▲ and ■——■, nephrectomy plus intravenous injection of GOT extract; ●——●, nephrectomy without injectoin of GOT; ○——○, laparotomy plus intravenous injection of GOT extract).

the experiment a free flow of bile through the fistula was maintained and no increase in serum bilirubin was noted. Before injection of GOT extract the serum GOT rose to 149 and 175 units/ml./minute respectively as the result of the surgical procedure. The 24-hour biliary excretion of GOT amounted to 1,260 and 1,612 units. After intravenous injection of approximately one million units GOT, the bile GOT activity bore no relationship to the serum GOT activity. In Experiment 1 the excretion of GOT during the first 24 hours after injection was slightly higher than the base-line excretion; in Experiment 2 no such increase was found. In both experiments the biliary excretion during the 3 days following the intravenous injection could in no way account for the more than one million units of injected GOT which disappeared from the circulation during this time.

In 3 additional experiments similar results were obtained. In all 5 experiments the concentration of GOT in gall bladder bile was from 8 to 21 times higher than that of the bile collected from the fistulas.

No inactivation of the enzyme in bile was found. The activity of gall bladder bile GOT showed a decay of less than 5 per cent after 24-hour incubation at room temperature. GOT was added in equal amounts in vitro to fistula bile and to phosphate buffer. Nearly identical values of GOT activity were found in the bile and in the buffer samples before and after 24-hour incubation at 37° C.

In order to test for a possible dissociation of the coenzyme in the process of hepatic excretion, the coenzyme, pyridoxal-5-phosphate (in concentrations ranging from 0.5 to 2 mg. per ml. bile), was added to both fistula and gall bladder bile samples. In no instance was any significant change in the GOT activity found.

DISCUSSION

All evidence presently available indicates that the tissue GOT and the GOT found in the serum following tissue necrosis are identical. The intravenous injection of GOT concentrates prepared from tissues thus simulates the release of endogenous GOT from necrotic tissues. After the intravenous injection of the crude GOT extracts used in this study, disappearance curves were obtained which were similar to those obtained by Fleisher and associates[6] after the intravenous injection of purified GOT concentrates. These authors have pointed out that the disappearance is much more rapid at the beginning. The inverse relationship between serum GOT and thoracic duct lymph GOT during the first 6 hours after intravenous injection of GOT extract found in our experiments offers an explanation for this phenomenon and indicates a diffusion of GOT into the extracellular fluid volume. Although thoracic duct lymph may not represent true interstitial fluid, the equilibration level of the serum and lymph GOT in each of 5 experiments was approximately one-fourth of the immediate postinjection serum GOT level which is considered strong evidence of a distribution of the injected enzyme in the entire extracellular fluid compartment.

The diffusion of GOT into the interstitial fluid was likewise observed following experimental myocardial infarction. The rise in lymph GOT paralleled

the rise in the serum GOT but remained at a slightly lower level for the first 32 hours. This was probably due to the prolonged release of GOT into the blood stream from the infarcted tissue. The distribution of the enzyme in the entire extracellular fluid volume and its simultaneous excretion or destruction accounts for the relatively small rise of serum GOT seen after the release of large amounts of GOT from the infarcted myocardium.

In view of the absence of the enzyme in the urine in spite of its free diffusion into the extravascular fluid, the possibility of an inactivation of the enzyme in the kidneys was entertained, but the experiments in nephrectomized animals ruled out such a mechanism. It remains undecided whether the absence of GOT in the urine is due to the impermeability of the glomeruli or due to complete tubular reabsorption. The former appears more likely because of the large size of the GOT molecule.[7]

Since high GOT activity was found in human gall bladder bile by Chinsky,[8] the biliary excretion was thought to play a prominent role in the elimination of the enzyme from the blood stream. In our experiments the GOT activity in gall bladder bile was 8 to 21 times higher than in liver bile. Although this seems to exceed the usually accepted concentrating power of the gall bladder, no other explanation can be offered.

The base-line excretion of GOT in bile was from 1,000 to 3,000 units per 24 hours in dogs weighing between 10 and 13 kilograms. The extracellular fluid volume in these animals was approximately 2,000 ml. and averaged 15 GOT units per milliliter. The pool of GOT normally present in the extracellular fluid is, therefore, in the order of 30,000 units, and an improbable slow turnover of the enzyme would occur if biliary excretion were the sole or principal mode of GOT elimination. Following experimental elevation of the serum GOT by intravenous injection of the GOT extract, the biliary excretion of GOT could not account for the disappearance of the large quantity of the enzyme from the blood stream. No correlation was found between the serum GOT activity and the concentration and excretion rate of the enzyme in the bile. In view of the latter findings the question can be raised whether the GOT found in bile actually represents GOT which is removed by the liver from the blood stream or whether the biliary GOT is formed in and secreted by the parenchymal cells of the liver.

SUMMARY

Following intravenous injection of GOT, three-fourths of the injected enzyme disappeared from the blood stream within 6 hours. The remaining one-fourth was cleared within 20 to 72 hours depending on the amount injected. The rapid disappearance phase was due to a diffusion of the enzyme into the interstitial fluid. Equilibration of serum and lymph GOT was reached within 6 to 8 hours following intravenous injection of GOT and marked the end of the rapid disappearance phase. A diffusion of GOT from the blood into the interstitial fluid was also found following myocardial infarction and hepatocellular necrosis. After intravenous injection of GOT no GOT elevations were seen in

the spinal fluid and no enzyme appeared in the urine. Identical disappearance rates were found in nephrectomized animals and in sham operated controls indicating that no inactivation of the enzyme occurred in the kidneys. The biliary excretion of GOT in the dog amounted to from 1,000 to 2,000 units per 24 hours. Following experimental elevation of the serum GOT by intravenous injection of the enzyme, no correlation was found between serum GOT level and the concentration and total excretion of the enzyme in the bile.

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HISTOPLASMOSIS AS A PROBLEM IN TUBERCULOSIS SANATORIUMS THROUGHOUT THE UNITED STATES

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SEROLOGIC surveys for histoplasmosis have now been completed in 22 tuberculosis sanatoriums located in 15 states extending from New York on the east to Arizona on the west. Most of the sanatoriums included in the study are located in the histoplasmosis belt of central United States.

MATERIALS AND METHODS

Initial serologic studies for histoplasmosis were performed on all patients in each sanatorium and continuing studies on all subsequent admissions are being carried out. In all instances a complement fixation test employing Histoplasmin as the antigen was performed. In later studies a yeast phase antigen was also routinely used. In one sanatorium in Missouri both a precipitin and a complement fixation test employing yeast phase antigen prepared by Dr. Samuel Salvin* of the Rocky Mountain Laboratories were employed. In several sanatoriums complement fixation tests were also performed for blastomycosis and coccidioidomycosis. The techniques and preparation of antigens have been described by various authors.[1] In all the complement fixation tests a titer of 1:8 or greater was considered positive except for coccidioidomycosis in which a titer of 1:4 or greater was considered positive. A titer of 1:40 or greater was considered positive on the precipitin test.

TABLE I. RESULTS OF COMPLEMENT FIXATION TESTS FOR HISTOPLASMOSIS ON 9,974 PATIENTS IN 22 SANATORIUMS IN 15 STATES (HISTOPLASMIN ANTIGEN ONLY)

STATE	NUMBER OF SANATORIUMS	TESTED		
		TOTAL	POSITIVE	PER CENT
Arkansas	2	2,718	47	1.7
Arizona	2	206	3	1.5
Illinois	1	315	9	2.9
Indiana	2	221	4	1.8
Iowa	1	306	3	1.0
Kentucky	2	469	16	3.2
Kansas	2	458	7	1.5
Louisiana	1	253	0	0.0
Maryland	1	368	5	1.4
Mississippi	1	59	3	5.1
Missouri	2	3,527	86	2.4
Nebraska	1	75	6	8.0
New York	1	305	3	1.0
Ohio	1	330	4	1.2
Oklahoma	2	164	0	0.0
Totals	22	9,774	196	2.0

RESULTS

A total of 9,774 sera from patients in 22 sanatoriums were tested for complement-fixing antibodies employing Histoplasmin as the antigen. Results of

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Received for publication July 20, 1957.

*Appreciation is expressed to Dr. Salvin for his assistance in these studies.

this test are tabulated in Table I by state. Of the sera examined it should be noted that 196 or 2 per cent showed a positive titer. The percentage of reactors ranged from 0 per cent in Louisiana and Oklahoma to 8 per cent in Nebraska.

Table II illustrates the results obtained when the yeast phase antigen was employed in the complement fixation test for histoplasmosis. The 5,311 sera examined by this test were obtained from the same patients included in Table I. It should be noted that 340 or 6.4 per cent were found to be positive using the yeast phase antigen. The over-all percentage positive should be noted to be greatly influenced by the Missouri results. In this particular group of patients the majority of the tests were performed by Dr. Samuel Salvin with a different antigen than was employed in the other tests. The exceedingly higher results obtained in several other sanatoriums using a recently prepared antigen led us to doubt the specificity of the later antigen.

TABLE II. RESULTS OF COMPLEMENT FIXATION TESTS FOR HISTOPLASMOSIS ON 5,311 PERSONS IN 9 SANATORIUMS (YEAST PHASE ANTIGEN ONLY)

STATE	NUMBER OF SANATORIUMS	TESTED		
		TOTAL	POSITIVE	PER CENT
Arkansas	1	1,047	77	7.3
Illinois	1	228	19	8.3
Indiana	1	71	27	38.0
Kentucky	1	351	62	17.6
Kansas	1	141	4	2.9
Maryland	1	25	6	24.0
Missouri	1	3,313	121	3.7
Nebraska	1	75	4	5.3
Ohio	1	60	20	33.3
Totals	9	5,311	340	6.4

TABLE III. COMPARISON OF INITIAL AND SUBSEQUENT SEROLOGIC STUDIES WITH THREE DIFFERENT ANTIGENS AT ONE SANATORIUM

	PER CENT POSITIVE		
	INITIAL SURVEY (702 PERSONS)	CONTINUED STUDY (2,611 PERSONS)	TOTAL (3,313 PERSONS)
Histoplasmin	1.9	2.6	2.5
Yeast phase	2.9	3.9	3.7
Precipitin	1.4	2.6	2.4
Total positive on one or more tests	5.4	6.5	6.3

Comparison of initial and continuing studies in one tuberculosis sanatorium in Missouri using all three serologic tests is presented in Table III. The purpose of this table is to compare the percentage of positive reactors in the initial survey and the continued study to the different antigens employed. The percentage of positive reactors with the Histoplasmin antigen totaled 2.5 per cent, being slightly higher in the continued study. The percentage of reactors was 3.7 per cent with yeast phase antigen and 2.4 per cent employing the precipitin test; again the continued study revealing a slightly higher percentage of reactors. It is, however, evident that with minor variations the initial and continued studies reveal rather consistent percentages of positive reactors. This table also illustrates that yeast phase and Histoplasmin complement fixation tests and the precipitin test all add valuable data to the possible serologic

response in such patients. It is also evidence that if all 3 serologic tests had been applied in the country-wide survey (Table I) more than 3 times as many positive reactions may have been found.

TABLE IV. COMPARISON OF YEAST PHASE AND HISTOPLASMIN ANTIGENS USED IN RECENT COMPLEMENT FIXATION TESTS ON 1,163 PERSONS

YEAST PHASE	HISTOPLASMIN	
	POSITIVE	NEGATIVE
Positive	23	125
Negative	8	1,007

As seen in Table IV, and mentioned previously, the yeast phase antigen recently employed in this study does seem to contain some elements of either oversensitivity or nonspecificity. This is illustrated by comparison of the results of tests with the two antigens on the same serum specimens. It is seen that 125 persons had a positive reaction by the yeast phase antigen and negative by the Histoplasmin antigen, as compared to 8 persons who had a positive reaction with the Histoplasmin antigen and negative with the yeast phase antigen. Twenty-three persons showed positive reactions to both antigens. Results such as this suggest different preparations of yeast phase antigen may vary in antigenic potency and that in the later series of tests some nonspecificity was evident.

TABLE V. RESULTS OF COMPLEMENT FIXATION TESTS FOR COCCIDIOIDOMYCOSIS ON 353 PATIENTS IN 2 STATES

	NUMBER TESTED	NUMBER POSITIVE
Arizona	189	1
Oklahoma	164	0
Total	353	1

Table V shows the prevalence of positive reactors to the complement fixation test for coccidioidomycosis among 353 patients in Arizona and Oklahoma sanatoriums. It was noted that a total of 353 sera was tested with only 1 positive, and this was from a patient in Arizona. The prevalence of positive serologic tests for coccidioidomycosis in patients in tuberculosis hospitals indicates that this may not constitute as big a problem as does histoplasmosis unless the patients in these sanatoriums were too carefully screened for the presence of tubercle bacilli prior to admission.

TABLE VI. RESULTS OF COMPLEMENT FIXATION TESTS FOR BLASTOMYCOSIS ON 624 PATIENTS IN 4 STATES

	NUMBER TESTED	NUMBER POSITIVE
Kentucky	293	7
Louisiana	253	0
Mississippi	59	2
Oklahoma	19	0
Total	624	9*

*Results of these sera on the histoplasmosis C.F. test: 6 higher titers to histoplasmosis; 1 equal titer to histoplasmosis; 2 lower titers to histoplasmosis.

In Table VI is shown the prevalence of positive complement fixation tests for blastomycosis among 624 sera in 4 different states. It is seen that a total of 9 sera showed a positive complement fixation test for blastomycosis. Of these 9 sera, 6 showed a higher titer on the histoplasmosis serologic tests. In one case the titers were equal and in only 2 instances did the titer for blastomycosis exceed that for histoplasmosis. These results indicated that in most of the instances these probably represent cross reactions with Histoplasmin antigen and particularly the yeast phase antigen of *Histoplasma capsulatum*.

DISCUSSION

The prevalence of positive reactors in the various sanatoriums throughout the central United States to the serologic tests for histoplasmosis indicates that this is a sizable problem to persons concerned with the control of tuberculosis. While the highest incidence was found in the histoplasmosis belt, positive reactors and confirmed cases have been uncovered throughout a major portion of the United States. Actually the prevalence of positive tests, if all 3 serologic tests are performed, is about 6 per cent. When one considers only the Histoplasmin antigen the percentage is as low as 2 per cent, and with the yeast phase antigen alone as high as 6 per cent. With the precipitin the results approximately equaled those with Histoplasmin. In the one sanatorium from the histoplasmosis belt where a continuing study has been made over 3 years with all these antigens, greater than 6 per cent of these patients were found to be positive.

Some of the difficulties of the employment of different antigens with the serologic tests are presented. It appears that further refinement of the yeast phase antigen is necessary as presently applied in our series. The precipitin test, originally found to be valuable, is no longer performed in many laboratories in the United States but does appear to have value. While it was shown that this test appears to offer little help in the diagnosis of cavitary histoplasmosis[2] it is frequently positive in other types of disease.[4]

It is of interest to note that with a minimum of effort approximately one-third of the serologic positives have been confirmed by cultural and/or pathologic demonstration of the organism. Our experience indicates that the higher the titer the more likely the patient has active histoplasmosis, and the greater the probability that the organism can be isolated if adequate cultural and pathologic examinations are undertaken. It should be pointed up that a negative serology does not exclude the diagnosis of cavitary histoplasmosis. Indeed, previous studies[2] have shown that between 10 and 20 per cent of these cases may not have demonstrable antibodies.

The general results obtained with the other antigens suggest that while the performance of coccidioidomycosis and blastomycosis serologic tests in conjunction with histoplasmosis tests offers valuable diagnostic information, it does not, in our opinion, contribute much in the way of positive results in central United States. Of course, from a practical point of view, a positive

serologic test for any one of the three antigens should lead to further diagnostic studies such as cultural and pathologic examination which should lead to the specific diagnosis.

SUMMARY

The serologic surveys reported in this paper present confirmatory evidence of the importance of histoplasmosis as a problem in tuberculosis sanatoriums in the United States. Employing the Histoplasmin complement fixation test 2 per cent of 9,774 patients were found to be positive, while employing the yeast phase antigen 6 per cent of 5,311 patients were found to be positive. The positives tended to occur in sanatoriums from Kansas to Maryland, with the higher prevalence in the sanatoriums in the high histoplasmosis belt. There is every evidence that the employment of additional antigens in the serologic tests would increase the yield of positive reactors.

Difficulties inherent in the serologic tests are discussed and some further study of the yeast phase antigen is indicated.

Cross reactions with other fungus antigens, such as Coccidioidin and Blastomycin, are reported and discussed.

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IMMUNOCYTOCHEMICAL STUDY OF PLASMA CELLS IN MULTIPLE MYELOMA

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INTRODUCTION

THE characteristic picture of plasmacytosis and hyperglobulinemia seen in multiple myeloma suggests the possibility that the neoplastic plasma cells may be the source of the high circulating levels of globulin in this disease. Miller and associates[1] have shown that the globulin extracted from plasma cells in multiple myeloma is electrophoretically similar to the serum globulin accounting for the hyperglobulinemia. That nonneoplastic plasma cells contain and presumably produce antibody, and hence gamma globulin, was suggested almost 50 years ago[2] and has been well demonstrated in recent years.[3-5] It is the purpose of this report to present direct evidence for the presence of gamma globulin in the cytoplasm of plasma cells in bone marrow and peripheral blood from human cases of multiple myeloma, using the "fluorescent antibody technique" of Coons and Kaplan.[6]

MATERIALS AND METHODS

Bone marrow aspirates and peripheral blood were obtained from 3 human cases of multiple myeloma. Thin smears were prepared and those used for immunochemical studies were air dried, stored in the refrigerator at 5° C., and stained within 24 hours. In some instances the dry smears were washed in buffered saline for 10 minutes prior to the fixation in alcohol in order to wash off excess plasma and avoid the possibility of nonspecific coating of cells with gamma globulin. The smears were stained with fluorescent antihuman gamma globulin (fl. anti-HGG) obtained from rabbits immunized with "normal" pooled human gamma globulin.* Controls for the specificity of localization of gamma globulin included staining with homologous fluorescent antihuman serum albumin (fl. anti-HSA) and heterologous fluorescent antigamma globulins (bovine and rabbit) as well as inhibition of specific staining with initial exposure to nonfluorescent anti-HGG.[7] The details of the technique employed for the preparation of fluorescent antisera†; the staining procedure and the optical system and photography were similar to those previously described.[7, 8] Parallel observations of a given field were done with both fluorescent and phase microscopy for the morphologic correlation of cells under study. Comparable smears were routinely stained with Wright's stain.

In order to compare the gamma globulin content of multiple myeloma plasma cells with their nonneoplastic prototype, plasma cells in spleens of hyperimmune rabbits were studied for their homologous gamma globulin and specific antibody content, also using the "fluorescent antibody technique."

From the Department of Pathology, University of Pittsburgh School of Medicine, Pittsburgh, Pa. (Publication No. 132).

This work was supported by research grant A 1195 (C2), United States Public Health Service.

Received for publication July 23, 1957.

*Poliomyelitis immune globulin, Lot C-420. Lederle Laboratories, Pearl River, N. Y.

†The fluorescein amine was obtained from Dr. Klaus Hofmann, Biochemistry Department, University of Pittsburgh School of Medicine.

RESULTS

Clinically, the 3 patients studied showed hyperglobulinemia with high levels of gamma globulin demonstrated by paper strip electrophoresis. Wright's stains of bone marrow smears showed the characteristic picture seen in multiple myeloma, with abundant single or clustered plasma cells, some of them of large proportions, others bizarre, with binucleation (Fig. 1). "Russell bodies" were not identified in these preparations. In one of the cases it was possible to observe in the peripheral blood smears an occasional plasma cell similar to those seen in bone marrow preparations. When similar smears were stained with fl. anti-HGG, a bright green, specific fluorescence was seen in the cytoplasm of plasma cells, indicating concentration of gamma globulin (Figs. 2, 3, and 4).

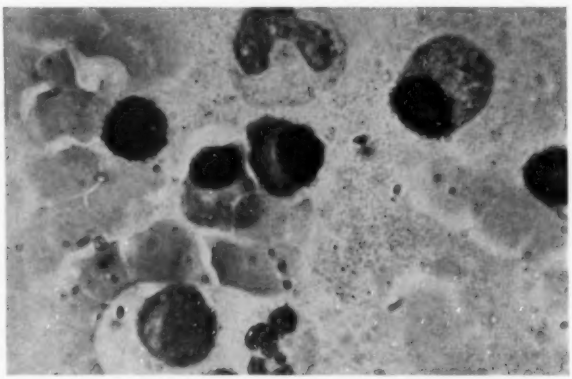

Fig. 1.—Bone marrow smear from patient with multiple myeloma to show plasma cells. Lymphocytes and cells of the myeloid series are also seen in this field. Scattered dots in the picture are artefacts. Wright's stain. (Magnification ×800; reduced ¼.)

The eccentrically placed nuclei of these plasma cells showed clearly by their lack of fluorescence. The gamma globulin present in these cells was uniformly dispersed throughout the cytoplasm. However, not all plasma cells showed similar intensity of fluorescence, suggesting a variation in the concentration of gamma globulin in these cells. Smears in which nonfluorescent anti-HGG was applied prior to the specific staining with fl. anti-HGG (control) showed less fluorescence indicating partial immunologic inhibition of staining of cytoplasmic gamma globulin by the previous exposure to the nonfluorescent anti-HGG. Likewise, staining with heterologous fluorescent antigamma globulins and homologous fl. anti-HSA (controls) failed to show fluorescence comparable to that seen when fl. anti-HGG was used. Sometimes polymorphonuclear leukocytes showed slight green fluorescence (Fig. 4) with both specific and control conjugates. This relatively nonspecific fluorescence of polymorphonuclear leukocytes has been observed previously[3] and was partially but not completely inhibited when the fluorescent conjugates were absorbed with chicken bone marrow powder.

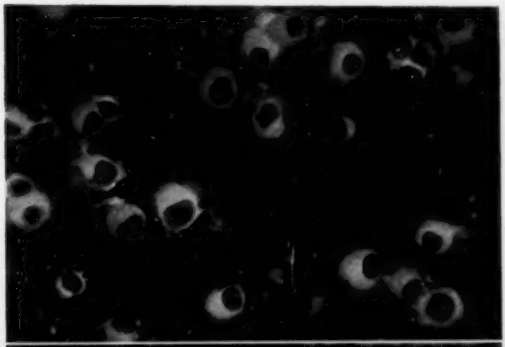

Fig. 2.

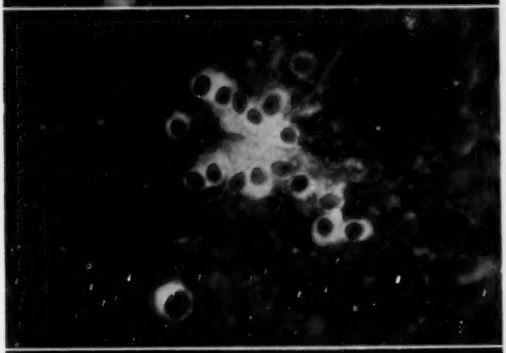

Fig. 3.

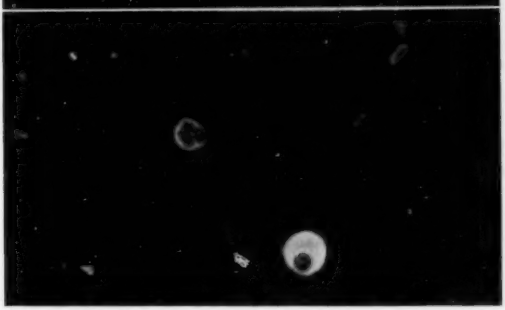

Fig. 4.

Fig. 2.—Lower magnification of a comparable smear to that seen in Fig. 1 stained with fl. anti-HGG. Note the bright specific cytoplasmic fluorescence of plasma cells, indicating concentration of gamma globulin. The nuclei stand out by their lack of fluorescence. The cytoplasmic fluorescence is not of the same intensity in all cells as seen particularly in a cell at the uppermost central portion of the picture. The red cells present in this field showed no fluorescence. Fluorescent micrograph. (Magnification ×320; reduced ⅓.)

Fig. 3.—Similar preparation to that seen in Fig. 2 stained with fl. anti-HGG to show clusters of plasma cells with specific cytoplasmic fluorescence. Fluorescent micrograph. (Magnification ×320; reduced ⅓.)

Fig. 4.—Peripheral blood smear from patient with multiple myeloma stained with fluorescent anti-HGG. Note the single plasma cell with bright specific fluorescence similar to that seen in Figs. 2 and 3. In the upper left quadrant, a polymorphonuclear leukocyte is seen showing "nonspecific" fluorescence of cytoplasm. Red cells in the field lacked fluorescence. Fluorescent micrograph. (Magnification ×320; reduced ⅓.)

When frozen sections of spleens of rabbits hyperimmunized with HGG or HSA were stained with fluorescent antirabbit gamma globulin (fl. anti-RGG) a specific green fluorescence was seen in the cytoplasm of plasma cells in the perifollicular zone, indicating the presence of homologous gamma globulin. In sections stained for the specific antibody, either anti-HGG or anti-HSA according to the "sandwich modification" of the fluorescent technique[3] the cytoplasm of the plasma cells was strongly positive (Fig. 5). However, no antigen was detected in these cells.

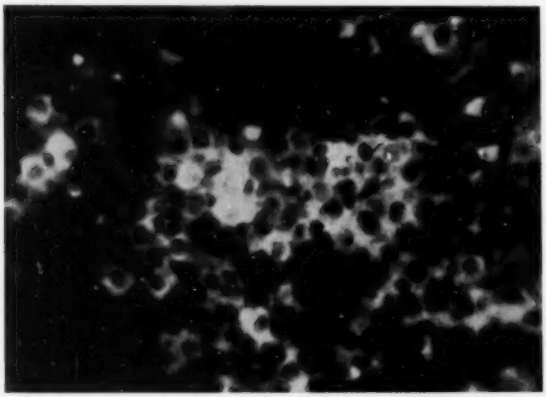

Fig. 5.—Section of spleen of rabbit immunized with human gamma globulin and stained with fluorescent anti-HGG according to the "sandwich modification of the fluorescent technique" for the detection of antibody. This field corresponds to the perifollicular zone of the splenic follicle. Note the bright specific cytoplasmic fluorescence of plasma cells, indicating concentration of specific antibody. Many cells morphologically resembling medium-sized lymphocytes also showed specific fluorescence but of somewhat lesser intensity. Fluorescent micrograph. (Magnification ×200; reduced ¼.)

DISCUSSION

The finding of specific concentration of human gamma globulin in the neoplastic plasma cells in multiple myeloma offers direct evidence in support of the concept that these cells are responsible for the high plasma levels of gamma globulin. It appears that plasma cells in these cases contain a gamma globulin immunologically identical with or similar (cross reacting) to normal human gamma globulin. Whether this gamma globulin is synthesized in the tumor cells or merely accumulated by them is not certain.

Recently, Rappaport and Johnson[9] in a morphologic and histochemical study of "Russell bodylike structures" in malignant lymphomas associated with acquired hemolytic anemias suggested that, under certain conditions, the neoplastic cells of malignant lymphomas are capable of synthesizing abnormal proteins and that these proteins may play an important part in the immunologic mechanisms responsible for the associated hemolytic anemia. White,[10] using the "fluorescent antibody technique," studied the nature of Russell bodies of plasma cells in the mouse spleen after intravenous injection of *Proteus vulgaris*. He concluded that his findings were compatible with the view that antibody was a major component of such Russell bodies.

The additional demonstration of specific antibody in the nonneoplastic plasma cells of hyperimmune animals supports previous observations made by Coons and associates.[3]

SUMMARY

By means of the "fluorescent antibody technique" it is shown that plasma cells from patients with multiple myeloma contain cytoplasmic concentrations of gamma globulin. Similarly, nonneoplastic plasma cells from hyperimmune animals showed concentrations of gamma globulin and specific antibody.

I wish to express my appreciation to Dr. Wallace N. Jensen and assistants, Department of Medicine, for the clinical study of these cases and to Mr. J. Arnold, Mr. N. Graver, and Miss J. Carnahan for their technical assistance.

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THE CONCEALMENT OF HEAVY BACTERIAL CONTAMINATION IN 25 PER CENT HUMAN SERUM ALBUMIN: ITS MECHANISM AND CLINICAL SIGNIFICANCE

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INTRODUCTION

NORMAL human serum albumin has become a useful adjunct in the management of a variety of diseases such as burns, shock, hypoproteinemia, hepatic cirrhosis, and acute pancreatitis.[1-4] In patients with rheumatic diseases and patients with hypersensitivity reactions clinical improvement has been noted following the daily intravenous administration of human serum albumin in small doses.[5, 6] The patients described in this paper were patients with various forms of arthritis.

This report is presented because 8 unfavorable reactions and one death have been observed following the intravenous use of human serum albumin. All of these reactions could be traced to bacterial contamination of the albumin, in spite of the fact that there was no gross detectable evidence that the material had been contaminated. The studies to be presented show that heavy bacterial growth may be present in 25 per cent human serum albumin without producing visible turbidity, formation of sediment, or color change. A systematic study was undertaken to explain the absence of visible turbidity in contaminated albumin solutions, and results of experiments and theoretical calculations are presented. These indicate that in solutions with the physical properties of 25 per cent serum albumin, visible turbidity cannot be expected unless the bacterial contamination is extremely heavy.

CASE REPORTS*

CASE 1.—The first case that came to our attention was that of M. B., a 28-year-old white woman with rheumatoid arthritis and keratodermia blennorrhagica. She had received daily intravenous injections of 25 per cent human serum albumin in doses varying between 10 and 20 ml. per day. No ill effect was noted during more than 2 months of such injections. On the seventy-third day of treatment, 8 hours after the albumin injection, the patient experienced a shaking chill, fever of 40° C., and severe headache. The clinical impression was that the patient had a septicemia. Blood cultures were taken and the patient given chloramphenicol. There was gradual defervescence over a period of 4 days. She made an uneventful recovery without sequelae.

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Received for publication July 27, 1957.

*We wish to express our appreciation to Dr. J. E. Ney, Dr. W. R. Merchant, and to the Staff of the George Washington University School of Medicine, at the George Washington University Hospital and the District of Columbia General Hospital, for permission to use their cases.

We did not see the patient ourselves, but were informed that a vial containing 100 ml. of human serum albumin had been entered daily for withdrawal of 10 ml. of the albumin, and had been replaced in the refrigerator shortly after each entry. The color of the remaining albumin was reported as unchanged and the solution as clear. On culture of this albumin growth of a paracolon bacillus was obtained. This organism was never recovered from the patient's blood.

CASES 2 to 7.—Several months later a group of 9 patients received 10 ml. each of human serum albumin between 9 and 11 A.M. of the same day. A single 100 ml. vial was used for all 9 patients. It had not been entered prior to 9 A.M. Later careful inquiry could detect no break in aseptic technique. Between 3 and 5 P.M. of the same afternoon 3 of the 9 patients reported that they were ill. The remaining 6 individuals of this group were contacted and either visited or called in for examination. Three of them were found to be ill and 3 showed no unfavorable reaction. The unaffected individuals had received the third, fifth, and ninth injections from the implicated bottle. The symptoms of the other 6 patients consisted of fever (2), headaches (6), nausea (6), vomiting (1), and a chilly sensation (3). None of the patients was seriously ill. The febrile patients were hospitalized for a few days and penicillin was given as a precautionary measure. They became afebrile in 24 and 36 hours and recovered uneventfully and without sequelae. In the other 4 patients the reaction was mild and subsided spontaneously and completely within 12 to 36 hours. Repeated blood cultures were done on all 9 individuals and all were negative. The majority of these patients was again placed on albumin therapy 1 to 8 weeks after their recovery. Individual 20 ml. vials were used and re-entry of vials was not permitted. No further untoward reactions were noted.

The remainder of the implicated human serum albumin was removed from the refrigerator and examined very carefully to detect even the slightest discoloration, sediment or turbidity and none were found. We could not find any defect in the glass or in the seals of the vial. Stained smears of this albumin showed a few gram-positive cocci. Heavy growth of *Micrococcus albus* was obtained on culture.

CASE 8.—The present study was begun because of these observations. While it was already in progress our attention was called to patient J. W. He was a 46-year-old white man with rheumatoid arthritis who had received human serum albumin intravenously in amounts of 10 ml. each on 3 successive days. The first 2 injections were tolerated well. One-half to one hour after the third injection the patient experienced severe multiple joint pain, chest pain, shortness of breath, abdominal pain, nausea, and vomiting. Temperature rose to 39° C. During the next few hours the patient had frequent shaking chills. Blood for culture was drawn immediately and the patient placed on chloramphenicol therapy. Later polymyxin and streptomycin were added. The patient was observed carefully for vascular collapse, so frequently observed in bacteremia due to gram-negative bacilli,[7, 8] but hypotension did not develop. He died 52 hours after the administration of the albumin. Permission for an autopsy was not granted.

We were notified of this patient's reaction and received blood for culture. This yielded *Proteus vulgaris*. We were also given the albumin left in the vial. Close inspection showed the bottle to be intact. The albumin showed no color change when compared with other bottles of the same lot, nor did it show even a trace of turbidity or sediment. Gram stain of the albumin showed gram-negative rods; heavy growth of *P. vulgaris* was obtained on culture. Questioning revealed that the bottle had been entered 3 times (2 times prior) for withdrawal of 10 ml. each of albumin and that it had been placed in a refrigerator shortly after each withdrawal.

Five milliliters of the contaminated albumin was placed in the incubator. The solution remained macroscopically unchanged for 6 days. After 7 days of incubation a minimal surface film developed which on smear showed gram-negative rods.

CASE 9.—Several months later we received a bottle of albumin that was thought to have caused a transient febrile reaction in a patient. Culture of this albumin revealed the presence of paracolon bacilli. Again there was no visible indication of contamination, and again there was history of previous entry of the vial. Blood cultures of this patient were negative.

Table I summarizes the clinical and laboratory data of the 9 patients.

TABLE I. SUMMARY OF CLINICAL AND LABORATORY FINDINGS

CASE NO.	CLINICAL REACTION	OUTCOME	ALBUMIN USED	VISIBLE EVIDENCE OF CONTAMINATION OF ALBUMIN	ORGANISMS DEMONSTRATED IN ALBUMIN BY SMEAR	ORGANISM ISOLATED FROM ALBUMIN BY CULTURE	ORGANISM ISOLATED FROM PATIENT'S BLOOD
1	Severe	Recovered	I	None	None	Paracolon bacillus	None
2 3 4 5 6 7	Mild Mild Mild Mild Moderate Moderate	Recovered	II*	None	Few gram-positive cocci	*Micrococcus albus*	None
8	Severe	Died	III	None	Many gram-negative rods	*P. vulgaris*	*P. vulgaris*
9	Moderate	Recovered	IV	None	None	Paracolon bacillus	None

*Three additional individuals received albumin No. II but had no adverse clinical reaction.

MATERIALS AND METHODS

Experimental.—Because of the apparent failure of human serum albumin to develop sediment or turbidity in the presence of bacterial contamination 25 per cent human serum albumin was inoculated with various bacteria and observed for development of bacterial growth and change in appearance. Ten organisms were employed: (1) *Micrococcus albus;* (2) *Micrococcus aureus;* (3) *Streptococcus faecalis;* (4) *Corynebacterium xerose;* (5) *Bacillus subtilis;* (6) *Escherichia coli;* (7) *Acrobacter aerogenes;* (8) *Proteus vulgaris;* (9) *Pseudomonas aeruginosa;* (10) *Candida albicans.* This group was chosen as representative of common air and skin contaminants.

One-tenth milliliter of a saline dilution of a 24-hour broth culture of the organisms was inoculated into 9.9 ml. of broth and 9.9 ml. of 25 per cent human serum albumin. The contents of the tubes were thoroughly mixed and a sample plated immediately in order to determine the exact size of the inoculum. The inocula varied slightly, but ranged between 10 and 40 organisms per milliliter of broth or of albumin. Observations on these cultures were made after 6, 24, 48 hours, one week, and two weeks of incubation at 37° C. Typical examples of the results are illustrated in Table II.

It was then determined how many organisms per milliliter are necessary to produce visible turbidity in nutrient broth, and how many organisms per milliliter are demonstrable by cultural means in human serum albumin without resulting in visible turbidity or formation of sediment.

A convenient means of estimating the number of bacteria per milliliter is to compare turbidity of bacterial cultures or suspensions with the McFarland Standard.[9] McFarland Standard No. 1 corresponds to a turbidity which is easily visible or to an optical density of 0.280 to 0.300 (Coleman spectrophotometer, model 14, reading at 650 mμ). For the purpose of this experiment McFarland Standard No. 1 was diluted tenfold. This standard

gives a spectrophotometer reading of approximately 0.030. Its turbidity corresponds to very minimal broth turbidity, just visible to the unaided eye, if examined closely and if compared with a clear control tube.

Broth cultures of the various organisms were made and observed at 30-minute intervals. As soon as a trace of visible turbidity appeared a sample was removed for bacterial counts by the serial dilution plate method and the remainder of the broth was used simultaneously for measurement of optical density in the spectrophotometer.

TABLE II. APPEARANCE OF BACTERIAL CULTURES IN BROTH AND IN 25 PER CENT HUMAN SERUM ALBUMIN

MICROORGANISM	TIME AFTER INOCULATION	TURBIDITY		SEDIMENT IN ALBUMIN	COLOR CHANGE IN ALBUMIN
		BROTH	ALBUMIN		
Esch. coli	0	0	0	0	None
	6 hours	+	0	0	None
	24 hours	++++	0	0	None
	48 hours	++++	0	0	None
	1 week	++++	0	Minimal	None
	2 weeks	++++	0	Minimal	None
M. albus	0	0	0	0	None
	6 hours	+	0	0	None
	24 hours	++++	0	0	None
	48 hours	++++	0	0	None
	1 week	++++	0	0	None
	2 weeks	++++	0	0	None

TABLE III. COMPARISON OF BACTERIAL COUNTS AND TURBIDITY IN BROTH AND 25 PER CENT ALBUMIN. (INCUBATION: 37° C.)

MICROORGANISMS	VIABLE ORGANISMS X 10^6		VISIBLE TURBIDITY		OPTICAL DENSITY*		APPEARANCE AFTER 1 WEEK'S INCUBATION
	BROTH†	ALBUMIN‡	BROTH	ALBUMIN	BROTH	ALBUMIN	ALBUMIN
C. albicans	0.7	0.2	+	0	0.038	0	
B. subtilis	3.5	19	+	0	0.030	0	Pellicle
C. xerose	3	36	+	0	0.035	0	
Str. faecalis	30	36	+	0	0.035	0	
M. albus	7	46	+	0	0.030	0	
M. aureus	10	47	+	0	0.030	0	
P. vulgaris	18	160	+	0	0.038	<0.015	
Esch. coli	16	400	+	0	0.030	<0.015	Slight sediment
A. aerogenes	7	540	+	0	0.030	<0.015	Slight sediment
Ps. aeruginosa	32	750	+	0	0.030	<0.015	Pellicle and pigment

*Increase of reading over uninoculated control.
†Counts made as soon as visible turbidity was noted and measured.
‡Highest counts obtained during entire period of observation.

Bacterial counts were also done on the human serum albumin which had been inoculated with the same organisms. The counts were done by serial dilution method 6, 24, 48 hours, and 1 and 2 weeks after inoculation. Table III illustrates that the albumin remained clear, even when bacterial counts in albumin showed 5 to 77 times as many organisms as were necessary to produce turbidity in broth. Sediment, if it occurred at all, developed late and was so slight that it was noticeable only on very close inspection. Growth of *B. subtilis* and *Ps. aeruginosa* could be detected by inspection of the tubes, as both produced a pellicle and the latter also caused greenish discoloration of the albumin in the upper third of the tube.

In the studies summarized in Tables II and III, small inocula, averaging from 10 to 40 organisms per milliliter, were used because in accidental contamination of blood or blood

products probably only few bacteria are introduced. Under the conditions of these experiments the highest viable bacterial counts in albumin were lower than those obtainable in suitable nutrient broth. Larger inocula were also used to determine whether these would result in heavier growth and in eventual turbidity of the albumin.

With larger inocula bacterial growth in the albumin was similar to that in nutrient broth although the rate of growth was slower. When such heavy growth was obtained in albumin, slight turbidity finally became discernible. Formation of sediment remained slight. Table IV represents typical results obtained.

TABLE IV. GROWTH OF *Esch. coli* (37° C.), USING INOCULA OF VARYING SIZES

INOCULUM NO. OF ORGANISMS PER ML.	HIGHEST BACTERIAL COUNT ORGANISMS/ML. × 10^6		TURBIDITY		SEDIMENT	
	BROTH	ALBUMIN	BROTH	ALBUMIN	BROTH	ALBUMIN
30	760	200	++++	0	+	+/− after 1 week
1,200	960	880	++++	+	+	+/− after 1 week

Three of the four contaminated vials of albumin had been entered repeatedly. Prior to and following entry they were stored in the refrigerator, having been kept at room temperature for an undetermined, though reportedly short, period of time. Albumin, inoculated with the test organisms was therefore incubated at temperatures of 4°, 27°, and 37° C. Table V illustrates results obtained. It will be noted that none of the microorganisms was cryophilic. The majority of the organisms grew better at 27° C. than at 37° C. Turbidity developed only in the albumin inoculated with *E. coli* and *A. aerogenes* at bacterial counts of 1.3 billion and 900 million, respectively.

TABLE V. GROWTH OF SMALL INOCULA (10 TO 40 ORG./ML.) IN 25 PER CENT HUMAN SERUM ALBUMIN AT 37° C., 27° C., AND 4° C.

MICROORGANISM	VIABLE COUNTS*		
	× 10^6 37° C.	× 10^6 27° C.	4° C.
C. albicans	0.2	1	No growth
B. subtilis	19	31	No growth
C. xerose	36	71	No growth
Str. faecalis	36	37	870 org./ml.
M. albus	46	113	No growth
M. aureus	47	49	No growth
P. vulgaris	160	520	No growth
Esch. coli	400	1,300†	No growth
A. aerogenes	540	900†	No growth
Ps. aeruginosa	750	860	No growth

*Highest count obtained during 2 weeks observation.
†Slight turbidity. All other tubes were clear.

Attempts were made to determine the factors influencing the observed phenomena. The high viscosity and specific gravity of 25 per cent albumin would account for the minimal and slow development of bacterial sediment. Systematic studies were undertaken in an effort to understand the absence of visible turbidity, and consideration was given to the effect of light absorption, bacterial concentration, index of refraction, and particle size.

Method.—In the subsequent experiments bacteria were suspended rather than grown in the media under study so that their size, shape, and concentration would vary as little as possible. The use of bacterial suspensions also made it possible to use substances with known physical properties that are not suitable for cultivation of microorganisms.

M. albus (No. 9491, American Type Culture Collection) was used as the test organism. It was grown in liquid or on solid media. After 3 washings in saline the organisms were

resuspended in 0.85 per cent saline, in concentrations corresponding to approximately McFarland Standard 1, 2, or 3. These stock suspensions were mixed thoroughly, aliquots centrifuged and the sediment resuspended in equal amounts of the test solutions, e.g., physiologic saline, 25 per cent albumin, or broth. Bacterial concentration in the stock and test suspensions was determined by the serial dilution plate method and by direct counts using the Petroff-Hausser counting chamber. Direct counts averaged approximately 33 per cent higher than viable counts. Turbidity and optical density of the bacterial suspensions were measured against the uncontaminated medium as a standard. All counts and measurements were done repeatedly because of the possibility of a considerable margin of error in a single experiment due to various factors, such as settling out of heavy bacterial suspensions in broth and saline, or difficulties in obtaining even bacterial distribution in the higher albumin concentrations.

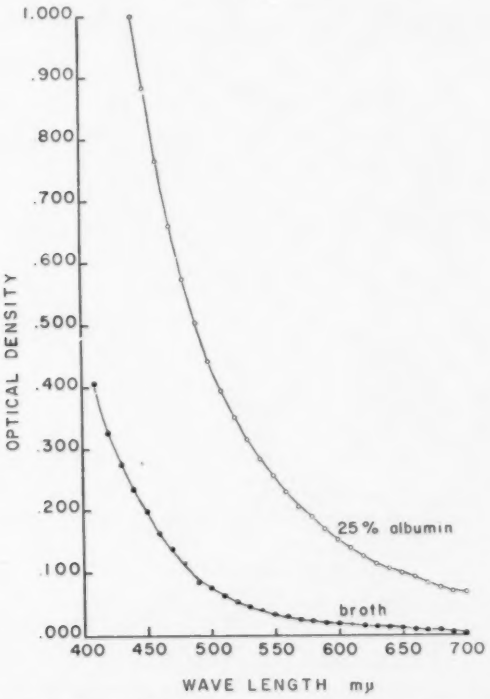

Fig. 1.—Absorption spectra of broth and 25 per cent human serum albumin at wave lengths of the visible range.

Light absorption: Studies showed that at wave lengths of 600 to 700 mμ the primary absorption of 25 per cent albumin is less than at the shorter wave lengths of the visible range. Also, in the long wave length range there is less variation of optical density per unit change in wave length than there is at the shorter wave lengths as shown in Fig. 1. In order to use these two factors to advantage a wave length of 650 mμ (using the Coleman spectrophotometer) was chosen to measure the optical density of the bacterial suspensions. This primary absorption of light by 25 per cent albumin over the entire visible range explains, though not fully, the decrease of visible turbidity of bacterial suspensions under ordinary white light, because of the reduction of intensity of the transmitted and scattered light.

Optical density of bacterial suspensions of known concentration was determined in various concentrations of albumin solutions. Composite results of 5 experiments are shown in Fig. 2. It can be seen that the optical density increases in an approximately linear fashion as a function of bacterial concentration. The slight deviation from linearity in the saline and 5 per cent albumin solution is a well-known phenomenon as optical density becomes large. It is also noted that the proportionality constant is dependent upon the concentration of the albumin as the optical density is only approximately 1/10 as great in the 25 per cent albumin as in the salt solution for the same bacterial concentration, e.g., the optical density of 200 million organisms per milliliter was 0.310 in saline, 0.210 in 5 per cent albumin, 0.090 in 15 per cent albumin, and 0.030 in 25 per cent albumin.

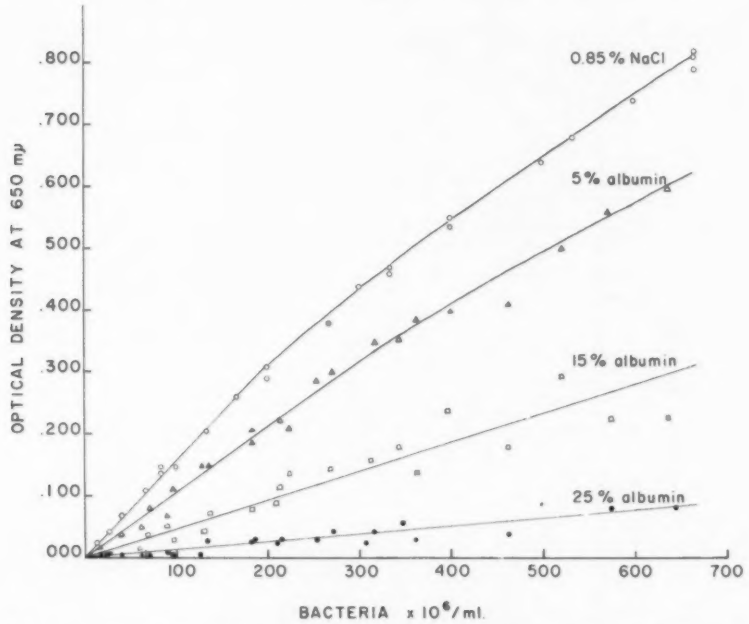

Fig. 2.—Observed optical density of increasing concentrations of bacteria suspended in saline and albumin solutions.

Index of refraction: Visible turbidity in a colloidal suspension is due to the differential scattering of white light by the small molecules of the dispersion medium and the larger particles suspended in it. The intensity of the scattered light is dependent on the difference in the refractive indices of the dispersion medium and of the suspended particle, and on particle size. If the disperse phase has an index of refraction identical with that of the dispersion medium, no turbidity should be observed when the former is suspended in the latter. Thus the lack of visible turbidity in 25 per cent albumin could be explained if bacteria and albumin had the same index of refraction.

Experiments were done to determine whether or not this was the case. The refractive index of bacteria has been reported to be in the range of 1.33 to 1.40[10]; the index of refraction of 0.85 per cent NaCl solution is 1.3345 and that of 25 per cent albumin is 1.3853.

Solutions representing a range of indices of refraction were prepared, including two with an index identical to that of 25 per cent albumin, e.g., 33 per cent sucrose and 37 per

cent glycerin. Visible turbidity and optical density of aliquots of bacterial suspensions in these solutions was determined.

It will be noted that visible turbidity in the sucrose and glycerin, though less pronounced than in the saline, was not diminished as markedly as it was in the 25 per cent albumin. Measurement of optical density showed that bacterial suspensions which produced an optical density of 0.270 in saline, produced an optical density of 0.160 in the glycerin, 0.090 in the sucrose, and 0.020 in the 25 per cent albumin; e.g., optical density in glycerin was approximately ½, in sucrose ⅓, and in albumin 1/10 of that of an aliquot of bacteria suspended in saline. Therefore, the explanation of the lack of visible turbidity did not appear to be a simple one and measurements and calculations were undertaken in an effort to explain these observations.

Theoretical calculations were made to get an estimated value of the optical density to be expected in the systems employed. From a consideration of the Rayleigh theory of light scattering, modified to take into account the fact that the size of *M. albus* is about the same order of magnitude as the wave length of light, one obtains the following expression for the optical density (O.D.):

$$\text{O.D.} = \frac{128}{3} \cdot \frac{\pi^5}{\lambda_0^4} \cdot R_0^6 \cdot \frac{n_m^4 \, (n_p^2 - n_m^2)^2}{(n_p^2 + 2n_m^2)^2} \, v \cdot S \, (x) \qquad (1)$$

where R_0 = radius of the suspended particle; n_m = index of refraction of the medium; n_p = index of refraction of the particle; v = number of particles per milliliter; $S(x)$ = geometrical factor depending on size and shape of the suspended particle.

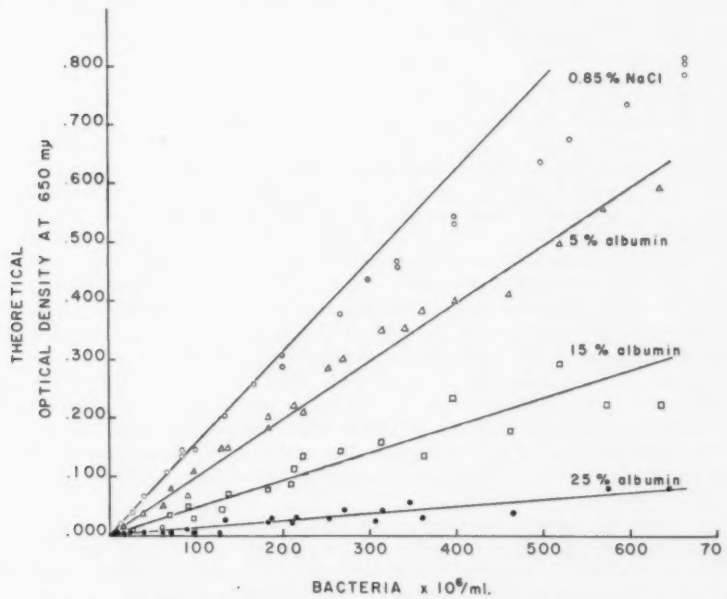

Fig. 3.—Comparison of the theoretically calculated optical density values (straight lines) and the observed optical density values (experimental points), presented in Fig. 2. Increasing concentrations of bacteria suspended in saline and albumin solutions.

These calculations were carried out over a range of indices of refraction (n_p 1.38 to 1.45) and particle size (diameter 0.6 to 1.2 microns) for *M. albus*. It was found that the absolute values of the calculated optical densities were very close to the experimentally observed ones, when the particle size was assumed to be 0.8 microns and the index of refraction was

1.4070. This is illustrated in Fig. 3, where the straight lines are the calculated optical densities for n_p 1.4070 and diameter 0.8 microns, and the points are the same experimental points shown in Fig. 2.

Since these values of particle size and index of refraction are entirely reasonable for *M. albus*, it seems from this study that the size of bacteria or behavior such as "clumping" are the same in the albumin and saline. The lack of effect of albumin solutions on the size of infectious agents has been demonstrated previously.[11]

TABLE VI. TURBIDITY OF SUSPENSIONS OF ALIQUOTS OF WASHED BACTERIA IN LIQUIDS WITH DIFFERENT REFRACTIVE INDICES

MEDIUM	PER CENT (W/V)	REFRACTIVE INDEX OF MEDIUM* (±0.0005)	VISIBLE TURBIDITY	O.D.† AT 650 mμ ± 0.010
Water		1.3325	++++	0.275
NaCl	0.85	1.3345	++++	0.275
Broth		1.3367	++++	0.280
Albumin	5.0	1.3450	++++	0.220
Albumin	15.0	1.3630	+	0.087
Gelatin	30.0	1.3845	+/−	0.032
Sucrose	32.5	1.3848	+	0.084
Glycerin	37.0	1.3852	++	0.160
Albumin	25.0	1.3853	+/−	0.020
Sucrose	33.0	1.3855	+	0.090
Albumin	36.0	1.4080	0	0.000

*Determined at room temperature.
†Increase over control.

As a further check on the calculated values of particle size and index of refraction of the bacteria, an additional observation was made. From a consideration that the index of refraction of albumin is a linear function of its concentration and from the measurements presented in Fig. 2, we estimated that a bacterial suspension in 36 per cent albumin (index of refraction 1.4080) would exhibit no visible turbidity. If this were found to be the case, it would give us an estimate of the index of refraction of *M. albus*. A sample of 25 per cent albumin solution was concentrated to 36 per cent in a vacuum desiccator at room temperature. *M. albus* was suspended in this solution in the same concentration that caused minimal visible turbidity in 25 per cent albumin. There was no visible turbidity nor was there measurable optical density in the 36 per cent solution. The above bacterial concentration in the 36 per cent albumin had to be increased by a factor of at least 5 to result in measurable optical density, and by a factor of 10 to result in minimal visible turbidity.

These experimental data allow an estimate of the refractive index of *M. albus*. The refractive index of *M. albus* is greater than that of 25 per cent albumin, e.g. greater than 1.3853. It is estimated that it approximates 1.4080, the refractive index of 36 per cent albumin. This experimental value is in good agreement with the theoretical value of 1.4070 used in equation 1. It is estimated that the strain of *M. albus* used in this study has a refractive index of 1.4070 ± 0.002.

The small difference between the index of refraction of *M. albus* and of 25 per cent albumin appears to be the primary reason for the low intensity of scattered light in contaminated albumin solutions as measured by optical density. In addition visible turbidity is reduced in this solution by its property of primary absorption of white light.

DISCUSSION

These clinical and laboratory observations are reported to caution against any procedure that may lead to contamination of 25 per cent normal human serum albumin by re-entry of vials. They also seem of interest because they demonstrate some of the properties of the albumin solution.

There have been a number of reports on transfusion reactions due to contaminated blood or plasma.[12-17] Detection of bacterial contamination in stored blood is a problem for which no satisfactory solution has been found.[12] During early clinical trials of individual preparations of serum albumin, Janeway and his co-workers[1] noted pyrogenic reactions with unsatisfactorily processed material, one of them "presumably due to bacterial contamination of a single bottle." The authors concluded that properly processed material does not produce reactions. To our knowledge there are no other cases reported where adverse reactions or death are attributed to contaminated human serum albumin. It is not generally realized that detection of bacterial contamination of albumin also presents a considerable problem.

A 25 per cent solution of human serum albumin is a clear straw-colored liquid. On standing at very high temperatures a haze with or without visible flocs appears. As this indicates incipient denaturation of protein, the initial turbidity of albumin solution and its rate of change at high temperatures (50 and 57° C.) were made the criteria of acceptability of human serum albumin solutions for the armed forces.[18]

To the clinician clarity of the solutions usually indicates not only stability of the product but also its freedom from bacterial contamination. However, since it has been shown here that human serum albumin does not cloud when contaminated, unless the contamination is very heavy, we wish to emphasize the necessity for strict adherence to the rule: "Discard bottle after it has once been entered."

The cases reported in this paper occurred in 3 different hospitals. The albumin used was from 2 different sources. This would indicate that accidental contamination of albumin is not an unusual occurrence. In two instances other vials of albumin of the same lot number (II and III) were available for examination and were found to be sterile. The same lots had been used clinically without untoward effect. The presence of bacteria in the one vial of albumin (II) that had never been entered prior to use remains unexplained. In the other cases there can be little doubt that bacteria were introduced by entry of the albumin vial.

In addition to the standard 100 ml. double-ended vial, normal serum albumin is also available in 50 ml. and 20 ml. vials for use in children or others who require less than 100 ml. per dose. In spite of the higher cost of the smaller vials their use in these cases is recommended, as bacterial contamination by re-entry seems unavoidable regardless of proper technique. The possibility of bacterial growth is not eliminated by storage in the refrigerator. There is evidence that cryophile bacteria have been responsible for the more serious reactions due to transfusion of contaminated blood or plasma.[15]

The measurements of optical density and turbidity of bacterial suspensions and the theoretical calculations presented in this paper suggest that the concealment of visible turbidity in 25 per cent albumin is due to a combination of factors. Visible turbidity results from scattering of white light. It appears that, because of the small difference in refractive index of bacteria and 25 per

cent albumin the intensity of such light scattering is low. This factor would reduce optical density and visible turbidity to approximately one-tenth of the values obtained in saline solutions. Visible turbidity is reduced still further by the primary absorption of white light by the albumin. Experiments and calculations show that *M. albus* causes visible turbidity in saline at bacterial concentrations of approximately 10×10^6 organisms per ml., but that 25 per cent albumin must contain about 200×10^6 organisms per ml. in order to exhibit visible turbidity. This marked reduction of visible turbidity may vary depending on the size, shape and refractive index of the respective bacteria. This variation however will not affect the strong light absorption by the albumin and the decrease of visible turbidity of bacterial suspensions will always be considerable.

It is probable, that in accidental contamination of albumin with few bacteria, bacterial multiplication will rarely result in bacterial concentrations of 200×10^6 organisms per milliliter. Albumin No. IV (see Table I) which caused a febrile reaction, contained only 3×10^6 organisms per ml. In this study bacterial concentration in albumin solution, experimentally contaminated with *M. albus*, did not exceed 113×10^6 organisms per milliliter. This number of staphylococci, causing no visible turbidity in the albumin, though showing marked turbidity in saline or broth, is certainly of serious clinical significance.

SUMMARY

1. A report of 9 patients is presented, who had adverse reactions to intravenous serum albumin. There was one fatal case.

2. The albumin used showed no visible evidence of bacterial contamination but was found to contain microorganisms on culture. Three out of four vials had been entered repeatedly.

3. Bacteriologic studies are presented which illustrate that bacterial growth does not cause visible turbidity in 25 per cent human serum albumin, unless the growth is extremely heavy, exceeding 200×10^6 organisms per milliliter.

4. Experiments and theoretical calculations are presented which demonstrate that the lack of visible turbidity is due to the combined effect of particle size of the bacteria, effective refractive index of microorganisms and albumin, and the property of 25 per cent albumin to absorb white light.

5. Vials of 25 per cent human serum albumin should never be re-entered because of the lack of visible evidence of accidental bacterial contamination.

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INSULIN BINDING IN VITRO BY LEUKOCYTES FROM NORMAL AND DIABETIC SUBJECTS

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CLINICALLY, at least two common types of diabetes have been recognized: lipoplethoric (obese type) and insulin deficient (juvenile type).[1] The lipoplethoric diabetic patient is characterized by obesity, absence of ketosis upon withdrawal of insulin, and relative insulin resistance, while the insulin deficient diabetic patient develops ketosis rapidly following insulin deprivation and is insulin sensitive.[1] A basis for these clinical observations has been provided by the results of Wrenshall and associates[2] who found little or no extractable insulin in the pancreas of the insulin deficient patient while there was detectable insulin in the pancreas of the lipoplethoric patient. In addition, normal amounts of circulating insulin-like activity have been measured in the plasma of the lipoplethoric diabetic while no such activity was found in the plasma of the insulin deficient patient.[3, 4] The physiologic basis for the difference in the response to insulin observed in the various types of diabetics has never been adequately explained.

Stadie and associates[5] demonstrated that insulin combined rapidly and firmly with muscle and developed the concept of insulin binding to tissue as a prerequisite for the hormone's physiologic activity. There was an apparent correlation between the ability of a given tissue to bind and to respond physiologically to insulin.[6] Haugaard and co-workers[7] demonstrated that leukocytes obtained from normal rat and rabbit peritoneal exudates had a relatively high affinity for insulin. White blood cells from diabetic patients have been reported to respond to insulin in vitro although no similar effect was observed with white blood cells from normal individuals.[8, 9]

Therefore, it seemed of interest to compare in vitro the binding of insulin to white blood cells obtained from normal individuals and various types of diabetic patients. If the insulin resistance in the lipoplethoric type of diabetic is due to a failure of tissues to bind insulin, it might be anticipated that such a defect should be manifest in the white blood cell, if it is a representative insulin sensitive tissue. A similar abnormality might also be a feature of the insulin resistance observed in other situations where no definite etiology has been demonstrated, as in diabetes with hemochromatosis. Furthermore, insulin resistance has been reported in diabetes associated with lymphatic leukemia.[10] This might occur as a result of the greatly increased number of

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Received for publication Aug. 5, 1957.

white cells binding an inordinately large amount of the circulating insulin. The present studies were undertaken to investigate some of these possibilities.

METHODS AND MATERIALS

White blood cells were obtained according to the method of Skoog and Beck.[11] Venous blood was obtained from all individuals in the fasting state. In the case of the diabetic patients, both breakfast and insulin were withheld. Five milliliters of heparinized blood was added to 5 ml. of a solution of 6 per cent Fraction I (bovine fibrinogen)* in physiologic saline. To this mixture was added 0.1 ml. of insulin-I^{131}† resulting in a final insulin concentration of 250 microunits per milliliter (0.01 μg), which is approximately in the physiologic range.[3] The total amount of radioactivity added to the blood-Fraction I mixture was ascertained from an 0.1 ml. aliquot before the white blood cell separation was begun. Following sedimentation for 25 minutes, the supernatant containing the white blood cells was removed and centrifuged at 1,200 r.p.m. for 10 minutes. The white blood cells were then washed a minimum of 4 times by resuspension in 8.5 ml. of physiologic saline and centrifuged as above. The washed white blood cells were resuspended in physiologic saline to make a final volume of 1.1 ml. After aliquots were removed for white blood cell counts, the amount of insulin-I^{131} bound to 1 ml. of the white blood cell suspension was measured in a well-type scintillation counter. All determinations were done in duplicate. In some experiments 1 ml. of packed red cells was also assayed for bound radioactivity after appropriate washing. The adequacy of the washing was determined by measuring the amount of radioactivity in the supernatant from the last wash. The amount of insulin bound to the white blood cells was expressed as micrograms per 10^{10} cells.

Binding of albumin-I^{131}‡ was also measured according to the above procedure except for the following modification. It was necessary to use an amount of albumin equivalent in weight to 2.5 milliunits of insulin per milliliter because the initial radioactivity of the albumin was low. Parallel studies were also done using this larger amount of insulin for comparison with the albumin experiments.

The white blood counts were done in a 1:100 dilution using red cell pipettes. Duplicate pipettes were used for each sample and two chambers were counted for each pipette to provide the mean value for each sample. A dilution of 1:200 was used where the white blood count was expected to be unusually high.

Patients with the following diagnoses were studied: juvenile diabetes, obese diabetes, acromegaly with diabetes, hemochromatosis with diabetes, acute myelogenous leukemia, and chronic lymphatic leukemia with diabetes. Only leukemic patients whose white blood cell counts were elevated at the time of study were included.

RESULTS

Table I indicates the tenacity of the insulin binding since very little radioactivity was removed from the white blood cells following the fourth washing. This is consistent with the results of Haugaard, Haugaard, and Stadie[7] who found that the insulin bound to white blood cells could not be removed by washing. In contrast to the observations with white blood cells, no such combination of insulin and red blood cells was found (Table II).

*The fraction I (bovine fibrinogen) was purchased from The Armour Laboratories, Kankakee, Ill.

†The insulin-I^{131} was obtained from Abbott Laboratories and was dialyzed against distilled water for 24 hours prior to use.

‡Albumin-I^{131} was obtained from Abbott Laboratories, North Chicago, Ill.

TABLE I. EFFECT OF SERIAL WASHES IN REMOVNIG INSULIN-I^{131} BOUND TO WHITE BLOOD CELLS (NORMAL SUBJECT)

WASH NO.	C.P.M./ML. OVER BACKGROUND	
	Sample 1	Sample 2
1-3 (combined)	70	187
4	-3	4
5	2	0
6	2	0
1 ml. suspension W.B.C. after sixth wash	188	218

TABLE II. INSULIN-I^{131} BINDING IN ERYTHROCYTE AND LEUKOCYTE PREPARATIONS (NORMAL SUBJECT)

MATERIAL COUNTED	C.P.M./ML. OVER BACKGROUND (AFTER SIXTH WASH)	
	Sample 1	Sample 2
1 ml. packed R.B.C.	4	8
1 ml. suspension W.B.C.	188	218

The amounts of insulin bound to 10^{10} white blood cells in the various groups of patient studied are summarized in Table III. There were no significant differences between the normal individuals and the various types of diabetic patients.

TABLE III. INSULIN-I^{131} BOUND PER 10^{10} W.B.C. IN W.B.C. PREPARATIONS OBTAINED FROM VARIOUS DIAGNOSTIC GROUPS

GROUP	NO.	μG INSULIN BOUND PER 10^{10} W.B.C. MEAN ± S.E.M.
Normals	5	0.016 ± 0.0056
Juvenile diabetics	7	0.023 ± 0.0100
Obese diabetics	9	0.034 ± 0.0064
Hemochromatosis with diabetes	3	0.013
Acromegaly with diabetes	2	0.027
Leukemia (myelogenous)	2	0.015
Leukemia (lymphatic) with diabetes	1	0.002

TABLE IV. COMPARISON OF INSULIN-I^{131} AND ALBUMIN-I^{131} BINDING TO W.B.C. PREPARATIONS

SOURCE OF W.B.C.	AMOUNT BOUND	μG PER 10^{10} W.B.C.
	INSULIN-I^{131}	ALBUMIN I^{131}
Normal	0.028	0.003
Juvenile diabetics	0.063	0.003

Table IV summarizes the results obtained when an equivalent amount by weight of albumin-I^{131} was substituted for the insulin-$I.^{131}$ Although there was approximately 10 to 20 times more insulin bound to white blood cells than albumin when equivalent weights were added, this difference disappears when they are compared on a molar basis. Such lack of specificity of the binding of insulin to tissue has been reported.[12]

DISCUSSION

The absence of significant differences in the ability of white blood cells from several types of diabetic patients to bind insulin suggests that variations in binding are not the basis for the clinical variations observed. However, since the

white blood cells probably represent only a small per cent of the total "insulin sensitive" tissue in the body, it is still possible that muscle and adipose tissue behave differently from the white blood cells in respect to insulin binding. It is also conceivable that the binding of insulin is in itself not sufficient to produce the full physiologic response. This concept derives support from studies of a humoral insulin antagonist associated with diabetic acidosis where evidence has been presented that the antagonist does not interfere with the binding of insulin but prevents insulin action after binding has occurred.[12]

The marked difference in the binding of insulin between the white and red blood cells indicates that this property is not shared by all types of cells. It may be pertinent that an effect of insulin on the carbohydrate metabolism of the red blood cell has never been demonstrated.[13] Although at present the responsiveness of white blood cells to insulin is equivocal,[14] it has been shown that mammary, adipose, and muscle tissue do bind insulin and are known to be insulin sensitive, while brain does not bind the hormone and is unresponsive to it.[13, 15] The results of the studies of the binding of insulin-I^{131} and albumin-I^{131} to white blood cells are consistent with a lack of specificity in the binding of the hormone to tissues.[16]

Although there is an increase in the actual amount of insulin bound in leukemia because of the high white blood cell count the insulin resistance associated with a case of leukemia probably is not a result of the augmented binding. The binding of insulin on a unit cell basis in our case of lymphatic leukemia was the lowest observed. This suggests that most of the binding is done by the granular leukocytes.

The insulin resistance associated with hemochromatosis and acromegaly does not seem to be related to the binding of insulin to white blood cells, although the number of patients studied is small.

SUMMARY

The in vitro binding of insulin to white blood cells obtained from patients with various types of diabetes has been measured using insulin-I^{131} and compared to normal individuals. The diabetic patients were from the following groups: juvenile, obese, acromegalic, and hemochromatotic. Selected patients with leukemia were also studied. No significant differences were observed among any of these groups. On a molar basis albumin-I^{131} and insulin-I^{131} were bound to white blood cells in comparable amounts. No similar binding to red blood cells was observed. The clinical differences in the various types of diabetes do not seem to be related to the ability of the white blood cell, as a possible representative insulin responsive tissue, to bind insulin.

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GLUTATHIONE CONCENTRATION IN THE ERYTHROCYTES OF NORMAL INFANTS AND INFANTS SUFFERING FROM ERYTHROBLASTOSIS FETALIS

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IT IS a well-known fact that the severity of the disease process encountered in infants with hemolytic disease of the newborn, secondary to an Rh blood group incompatibility, varies greatly. This statement is valid even in infants of identical gestational age born to women with identical serologic findings. In spite of extensive investigation and improved immunoserologic and other techniques, there is still no definition of what factor(s) is (are) responsible for this variation. The study presented in this paper represents a further search as to the identity of this unknown factor. More specifically, erythrocyte glutathione levels of infants suffering from this disease were measured and compared with levels found in normal newborn infants.

METHODS AND MATERIALS

The method used for measuring the erythrocyte glutathione level in the blood of these infants was that of Patterson and Lazarow.[1]

Thirty-two normal infants and 31 infants suffering from hemolytic disease of the newborn were examined during their first 24 hours of life. The severity of disease encountered in the erythroblastotic infants varied from mild to severe. In addition, a number of normal infants were tested on the second, third, fourth, and fifth days of life. Eleven normal adults also were examined. The blood specimens were obtained by venepuncture or from the umbilical vein at the time of exchange transfusion. Five milliliters were drawn into a 10 ml. syringe, the dead air space of which was filled with Liguaemin.† The blood was then transferred to a clean test tube containing 2 to 3 drops of Liquaemin, the tube stoppered and inverted 2 to 3 times. The determinations were carried out within a few hours following collection.

RESULTS

The results are presented in Tables I, II, and III. The mean erythrocyte glutathione concentration of the 11 normal adults was 81 mg. per 100 ml. of erythrocytes with a standard deviation of the ±8.1 mg. and that of the 32 normal infants during the first 24 hours of life was 87 mg. per 100 ml. with a standard deviation of ±11.6. The mean value for the 31 erythroblastotic infants during the first 24 hours of life was 89.4 mg. per 100 ml. of

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This investigation was supported by Research Grant B-626 from the National Institute of Neurological Diseases and Blindness, of the National Institutes of Health, Public Health Service.

Received for publication Aug. 7, 1957.

Technical assistance was provided by Marian L. Jones.

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†Liquaemin (heparin solution, 1,000 units per 1.0 ml.), Organon, Inc., Orange, N. J.

erythrocytes with a standard deviation of ±15.3. A comparison of the mean values found in the series of normal adults, normal infants, and erythroblastotic infants did not reveal a significant difference.

TABLE I. GLUTATHIONE CONCENTRATIONS IN THE ERYTHROCYTES OF 11 NORMAL ADULTS, 32 NORMAL, AND 31 ERYTHROBLASTOTIC INFANTS
(Mg. per 100 c.c. of Erythrocytes)

NORMAL ADULTS	NORMAL INFANTS (FIRST 24 HOURS OF LIFE)	ERYTHROBLASTOTIC INFANTS (FIRST 24 HOURS OF LIFE)
85	106	74
73	71	97
77	69	83
64	80	70
83	71	94
79	79	81
92	81	92
81	90	91
76	73	85
89	71	82
76	76	94
89	93	77
94	93	80
	69	79
	97	69
	85	80
	86	100
	88	80
	86	76
	86	81
	79	80
	73	85
	98	87
	103	82
	102	85
	112	92
	93	91
	96	129
	100	103
	94	113
	80	159
	88	
Mean 81	Mean 87	Mean 89.4
S. D. ± 8.1	S. D. ± 11.6	S. D. ± 15.3

TABLE II. GLUTATHIONE CONCENTRATIONS IN THE ERYTHROCYTES OF NORMAL INFANTS, MILD ERYTHROBLASTOTIC INFANTS, AND SEVERE ERYTHROBLASTOTIC INFANTS
(Mg. per 100 c.c. of Erythrocytes)

NORMAL INFANTS	MILD ERYTHROBLASTOTIC INFANTS (FIRST 24 HOURS OF LIFE)	SEVERE ERYTHROBLASTOTIC INFANTS (FIRST 24 HOURS OF LIFE)
	74	92
	97	91
	83	87
	70	113
	94	82
See Table I	81	129
	92	103
	91	159
	85	85
	82	
	94	
Mean 87	Mean 86	Mean 105
S. D. ± 11.6	S. D. ± 8.3	S. D. ± 23.8

Eleven of the erythroblastotic infants represented mild cases who did well without the benefit of an exchange transfusion and 9 infants represented severe cases who required more than one exchange transfusion to control their plasma pigments during the first 72 hours of life below levels associated with the development of kernicterus. The mean values and the standard deviations of these two groups of infants are presented in Table II. The mean level in the mild group was 86 with a standard deviation of ±8.3 and that of the severe cases was 105 with a standard deviation of ±23.8. In spite of the fact that the difference between these two means is greater than two times their standard error there was some doubt as to whether this difference was significant. First, it was observed that the standard deviation encountered in the series of sicker infants was large. In addition, chance dictated that most of the sicker infants were encountered during the latter part of the study. Therefore the 32 normal infants were divided into two groups—those tested during the first half of the study and those tested during the second half. The means of these two subdivisions varied significantly, with the mean of the later determinations being a higher figure. This indicated a systematic error, perhaps reflecting a learning experience in technique. When the mean of the sicker erythroblastotic infants was compared to the mean of the normal infants tested during the second half of the study, no significant difference could be detected. Therefore, it appears reasonable to assume that no significant difference was demonstrated between the erythrocyte glutathione levels of normal, mildly affected, and severely affected erythroblastotic infants.

TABLE III. GLUTATHIONE CONCENTRATIONS OVER THE FIRST 5 DAYS OF LIFE IN NORMAL INFANTS
(Mg. per 100 c.c. of Erythrocytes)

0 TO 24 HOURS	25 TO 48 HOURS	49 TO 72 HOURS	73 TO 120 HOURS
	88	96	130
	74	82	73
See Table I	90	89	93
	86	83	77
	104	83	
		96	
		102	
		107	
		95	
		93	
		77	
		86	
Mean 87 S. D. ± 11.6	Mean 88 S. D. ± 9.6	Mean 91 S. D. ± 8.6	Mean 93 S. D. ± 22.5

A number of determinations were carried out in normal infants after their age of 24 hours. These results are presented in Table III. Again, no significant difference could be shown.

DISCUSSION

Recently Beutler and his associates[2] demonstrated a significantly reduced glutathione level in the erythrocytes of individuals who suffered hemolytic crises

when given the antimalarial drug, primaquine. These investigators hypothesized that the lower erythrocyte glutathione concentration rendered these individuals more susceptible to the hemolytic action of this drug. They suggested that hemolytic compounds damage the red cell by becoming oxidized and then oxidizing hemoglobin or stroma to form Heinz bodies with resultant hemolysis. Glutathione, which accounts for over 95 per cent of the reduced nonprotein sulphydryl compound in the red cell, is a reducing substance. It seemed possible, to Beutler and his associates, that glutathione might keep Heinz body-forming substances in the reduced state and thus prevent them from oxidizing hemoglobin or stroma. A deficiency of glutathione would, therefore, render the erythrocyte more susceptible to hemolysis.

In view of Beutler's findings, it seemed worth while to search for a similar defect in the antibody-coated erythrocytes of infants suffering from hemolytic disease of the newborn secondary to an Rh blood group incompatibility. As indicated, no significant difference between the glutathione levels of the erythrocytes of normal infants, mild erythroblastotic infants and severely affected erythroblastotic infants was demonstrated. A real contribution to the understanding of this disease will be made when the cause for the wide variation in the severity of hemolytic disease of the newborn is recognized. It is only partially explained by the maternal serologic findings and the state of maturity of the infant.

SUMMARY

In further pursuit of the reason for the variation in severity of erythroblastosis fetalis secondary to an Rh blood group incompatibility in infants of similar gestational age born to women with identical serologic findings, erythrocyte glutathione levels were determined on normal and erythroblastotic infants. The results obtained are presented. No significant differene was observed between the mean values found in normal and erythroblastotic infants.

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PROTECTIVE EFFECT OF COMPONENTS OF NORMAL BLOOD AGAINST THE LETHAL ACTION OF ENDOTOXIN

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IN RECENT years evidence has accumulated that certain components of the blood influence the activity of bacterial endotoxins. Thus, incubation of components of blood with endotoxin may alter: (1) the reaction of endotoxins with specific antibody, (2) the chemical structure of endotoxin, and (3) the pyrogenic action of endotoxin.

Cluff[1] found that incubation with normal rabbit serum altered the pattern of precipitation of endotoxin in agar gel containing specific antibodies to the endotoxin. Similarly, Stauch and Johnson[2] observed that incubation with normal serum reduced by 40 to 50 per cent the ability of purified *S. typhi* endotoxin to form precipitins with specific antisera.

That the chemical nature of endotoxin is altered by incubation with normal serum is indicated by the work of Rowley.[3, 4] In the course of studies of the effect of endotoxin on the bactericidal action of serum, he observed that normal serum could split off radioactive phosphorus that had been incorporated into purified *Esch. coli* lipopolysaccharide.[4] Evidence was presented that such action of normal serum in splitting phosphate from endotoxin is separable from the properdin system, as well as from serum alkaline phosphatase activity.[4]

Much of the evidence for an interaction between normal serum and endotoxin has been obtained from the demonstration that the pyrogenic activity of endotoxin is altered by normal serum. However, the results are confusing; both augmentation and attenuation of the febrile response have been observed. Thus, Goodale and associates[5] showed that, when pyrogenic doses of endotoxin were incubated with normal serum at 37° C. for 3 hours, the pyrogenic response in man was *attenuated*. Similarly, Hegemann[6] found that the pyrogenic effect in man of filtrates of *Esch. coli* vaccine was *diminished* after incubation for 3 hours at 37° C. with citrated or heparinized human plasma or serum.

On the other hand, many observations have indicated that components of the blood may *augment* the febrile response in rabbits. Farr and LeQuire[7] found that, when a suspension of killed *S. typhi* was incubated with citrated rabbit plasma before injection into rabbits, the fever that was produced appeared sooner and was longer in duration than in animals receiving vaccine alone. These observations were extended by Grant and Whalen[8] and by Farr and co-workers[9] who found that not only citrated rabbit plasma but also rabbit

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This study was aided by Research Grant E23 from the National Institutes of Health.
Received for publication Aug. 5, 1957.

serum, heparinized rabbit plasma, normal human serum, and normal guinea pig serum displayed this augmenting effect. Highly purified as well as crude extracts of gram-negative bacteria could be used as pyrogens in this reaction.[9, 10] The augmenting effect was evident when the plasma or serum was incubated with pyrogen under diverse conditions, e.g., 5° C. for 12 to 18 hours, 37 to 29° C. for 2 to 3 hours, and at 37° C. for 2 to 15 minutes.[7, 9]

The reasons for the divergent actions of blood components on the pyrogenic response to endotoxins are not clear. Changes in the febrile response may be manifested by alteration of either the latent period or the duration and intensity of the fever. Thus the presence of large numbers of leukocytes in plasma or serum that has been incubated with pyrogen shortens the latent period,[11] presumably by the release of "endogenous pyrogen."[12, 13] However, the number of leukocytes necessary for this reaction (1.5 to 2.0 $\times$ 10^8) is so great that it is unlikely that all of the augmenting effects of serum that have been observed[7-10] can be explained on this basis. It has been suggested that normal blood may contain both inhibitors and augmentors of the febrile response.[9] The relative concentrations of these substances may, therefore, determine the ultimate effect of incubation on the activity of pyrogens.

There are, then, many lines of evidence that components of blood alter endotoxin chemically, antigenically, and in terms of ability to produce fever. It seemed likely that the lethal action of endotoxin would also be affected by incubation with blood components. There seemed to be no basis for predicting whether such components would augment or decrease the lethal effect of endotoxin. This was true not only because of the conflicting evidence concerning the effect of blood components on the pyrogenic action of endotoxin, but also because lethal toxicity and pyrogenicity of endotoxins are separable qualities.[14]

In the experiments to be cited, it has been demonstrated that components of normal blood from several animal species attenuate the lethal action of endotoxin. Certain additional observations concerning the occurrence and activity of the attenuating substance are presented.

MATERIALS AND METHODS

Bacterial Vaccines and Endotoxins.—The organisms used were *S. typhi* (*Ty*2),* *S. typhi* (0901),† *S. typhimurium*,† and *Esch. coli* (0127:B8).† These were grown in a synthetic medium consisting of casein hydrolysate, fortified with tryptophane and yeast extract and grown according to the method of Formal and Baker.[15] The final suspensions contained approximately 10^8 to 10^9 organisms per milliliter. These suspensions were used immediately for the preparation of vaccines or were stored at −20° C. until needed.

Vaccines were prepared as follows: (1) Heat killed. The aliquot of bacterial suspension was placed in a 56° C. water bath for 30 minutes. On cooling, a sterility test was performed by inoculating a loopful of the heated suspension in broth, and the treated material was stored at 4° C. If no growth occurred in the inoculated broth, the treated suspension was dialyzed in running tap water at 12 to 14° C. for 16 to 24 hours, and in sterile physiologic saline at 4° C. for 4 to 6 hours. The pH was adjusted to neutrality with 0.1 N NaOH. (2) Chloroform killed. To about 100 ml. of bacterial suspension was added 1 per cent by

*Kindly provided by Dr. E. E. Baker, Boston University School of Medicine, to whom we are also indebted for much helpful assistance.

†Kindly provided by Difco Laboratories, Detroit 1, Mich.

volume of chloroform. The mixture was shaken manually for 5 minutes. Sterile air was then bubbled through the mixture at room temperature (25 to 27° C.) for 10 to 16 hours to eliminate the chloroform. The sterility was tested and the treated suspension dialyzed and brought to neutrality as above. (3) Phenol killed. One gram of phenol was added to 50 ml. of thawed bacterial suspension that had been stored at −20° C. After 3 hours at 25 to 27° C., the treated suspension was tested for sterility, dialyzed, and prepared as above. (4) Formalin killed. Formalin killed vaccines were prepared in the same manner as the phenol killed, except that 0.15 ml. of formalin was used instead of phenol. The toxicity of all vaccines was such that 0.8 to 1.6 ml. of vaccine given by the intracardiac route killed 47 to 90 per cent of 100 to 150 gram albino rats.

Trypsinized endotoxin was prepared by a method previously described.[16] Briefly, the procedure consisted of chloroform killing of the bacterial culture as above, followed by trypsin digestion and partial purification of the endotoxin by precipitation with acetone and alcohol-ether. The final solution was made up in neutral physiologic saline and heated at 56° C. for 30 minutes in a sterile vial. Two lots of trypsinized endotoxin were prepared using *S. typhi* (*Ty*[2]). The first lot contained 11.7 mg. per milliliter of active dry material. The LD_{50} by the method of Reed and Muench[17] was 0.05 ml. (0.59 mg.) per 100 gram rat and 0.15 ml. of this lot killed about 90 per cent of rats. The second lot contained 10.5 mg. per milliliter of active dry material; the LD_{50} was 0.06 ml. (0.63 mg.) per 100 gram rat, and 0.10 ml. killed about 80 per cent of rats.

Purified lipopolysaccharides from *S. typhi* (*0901*), *S. typhimurium*, and *Esch. coli* (*0127:B8*),* prepared according to the method of Boivin and associates,[18] using cold trichloroacetic acid extraction, were dissolved in sterile physiologic saline in a concentration of 2 mg. per milliliter, and titrated to neutrality. From 0.6 mg. to 2.0 mg. of these purified lipopolysaccharides killed 50 to 100 per cent of 100 to 150 gram albino rats so that the potency of the "purified lipopolysaccharides" was not markedly different from that of the trypsinized endotoxin.

Blood and Blood Cells.—Blood was obtained aseptically from normal human subjects by venipuncture, and from 2 to 3 kilogram male albino rabbits or 200 to 250 gram male albino rats by cardiac puncture. This was allowed to clot for 4 to 5 hours at 4° C. and the serum was separated by centrifugation at 2,000 r.p.m. The serum was used immediately or stored at −20° C. Whole rat blood was prevented from clotting by the addition of 0.05 ml. heparin per 15 ml. blood. It was used within 4 hours of withdrawal. Dialyzed rabbit serum was obtained by dialysis in cellulose casing,† first against running tap water at 12 to 14° C. for 20 hours, then against distilled water at 4° C. for one hour, and finally against physiologic saline at 4° C. for 2 hours. The dialyzed serum was then reconstituted to its original volume with saline and used within 24 hours.

Leukocytes were harvested from the peritoneum of a rabbit after infusion of sterile physiologic saline according to the method of Bennett and Beeson.[12] About 120 ml. of peritoneal washings containing 2,500 leukocytes per c.mm. were allowed to stand at room temperature for one hour and were then centrifuged at 2,000 r.p.m. for 20 minutes. The supernatant was labeled "leukocyte supernate." The sediment was washed 3 times and suspended in saline to a concentration of about 6,000 leukocytes per c.mm. This was labeled "washed leukocytes."

To obtain washed red blood cells of the rat, pooled red cells were washed 3 times in 3 volumes of physiologic saline. The final saline suspension contained about 85 per cent red cells by volume.

Plasma and Plasma Fractions.‡—All of the human plasma and plasma protein fractions that were used came from a single lot (run 1014), except for Fraction III_0 (run C_1), Fraction "Super III_0," and properdin-free Fraction III (run M_1). Fractions I, II + III,

*Kindly supplied by Difco Laboratories, Detroit 1, Mich.

†Visking Corporation, Chicago, Ill.

‡Kindly supplied by Dr. R. B. Pennell, Blood Characterization and Preservation Laboratory, Protein Foundation, Inc., Boston, Mass., to whom we are also indebted for much helpful advice.

IV_1, IV_4, and V were prepared by method 6 of Cohn and co-workers.[19] These fractions contain, respectively, fibrinogen, beta and gamma globulins with lipoprotein, alpha and beta globulins with lipoprotein, and alpha and beta globulins without lipoprotein as the major components. The subfractions of Fraction II + III were prepared by the method of Oncley et al.[20] Fraction II contains gamma globulin, and Fraction III contains beta-1 lipoprotein, isoagglutinins, prothrombin, and plasminogen as the predominant components. Fraction III_0 consists largely of the beta-1 lipoprotein. Fraction "Super III_0" is the unanalyzed supernatant obtained in the precipitation of Fraction III_0 after separation from Fraction II + III.[20] Properdin was prepared by a modification of the method described by Pillemer and associates.[21] Properdin-free Fraction III was prepared from plasma that had been absorbed with zymosan.

Most of the fractions were obtained as dry powders and dissolved in physiologic saline on the day they were used. The concentrations of the dissolved protein fractions were equivalent to their concentrations in normal plasma[19, 20] and the pH was adjusted to neutrality with 0.1 N HCl or NaOH. Each fraction was allowed to dissolve at 25 to 27° C. for 45 minutes and was then centrifuged at 2,000 r.p.m. to remove insoluble sediment and floating material.

Serologic Studies.—For the detection of antiendotoxin precipitins, 0.2 ml. of trypsinized endotoxin was added to 0.2 ml. twofold serial dilutions of serum, plasma, or plasma fraction. The tubes were incubated at 37° C. for 2 hours, and read after standing overnight at 4° C.

Sensitized red cells for hemagglutination were prepared from oxalated or defibrinated blood of normal rabbits. The plasma was drawn off, and the red cells washed 3 times, each time with 10 volumes of neutral physiologic saline. An equal volume of trypsinized endotoxin was added to the packed, washed cells and the mixture incubated at 37° C. for 2 hours. Unadsorbed endotoxin was removed by washing the sensitized red cells 3 times with about 10 volumes of physiologic saline. To 0.4 ml. of twofold serial dilutions of the solution to be titrated for antiendotoxin hemagglutinins was added 0.2 ml. of a 1 per cent suspension of sensitized red cells. The tubes were incubated at 37° C. for 2 hours, and allowed to stand overnight at 4° C. Hemagglutination was read macroscopically and microscopically. When human plasma was tested, initial adsorption with normal rabbit red cells was performed.

For certain studies it was desirable to adsorb hemagglutinins from Fraction II + III. Sensitized red cells (0.35 ml.) were added to 7 ml. of Fraction II + III. The mixture was incubated at 37° C. for one hour, then centrifuged at 2,000 r.p.m. and the sediment discarded. This process was repeated 3 times using fresh sensitized cells each time. After the first two adsorptions, the added red cells showed clumping, but by the third adsorption no clumping was evident. The adsorbed fraction, free of hemagglutinins, was used within 24 hours.

Experimental Animals.—Male albino rats* weighing 100 to 150 grams were used in these experiments. The substances in the test systems were injected by the intracardiac route as previously described.[16] The injection volume was between 0.07 and 2.0 ml. After injection of a lethal dose of endotoxin death generally occurred in the rats in 3 to 6 hours, and was preceded by ruffling of fur, lethargy, respiratory depression, paralysis of limbs, and convulsions. Rarely, a death occurred between 16 and 24 hours after injection of endotoxin and so mortality was recorded 24 hours after injection. Accidental deaths due to improper injection or to anesthesia occurred in less than 1 per cent of the animals used.

The toxicity of endotoxin to embryonated chick eggs has been described by Smith and Thomas.[22] The yolk sacs of 11-day-old embryonated eggs were inoculated with 0.1 to 0.4 ml. of a solution containing serial tenfold dilutions of endotoxin with or without other test substances. The eggs were candled daily and deaths occurring during the first 72 hours after injection were recorded. Initial studies had shown that maximal susceptibility to endotoxin was found in 11-day-old embryos.

*Obtained from Charles River Laboratories, Wilmington, Mass.

RESULTS

Protection of Rats Against the Lethal Action of Endotoxin by Blood and Its Components.—Whole rat blood, washed rat red cells, and serum or plasma from man, rabbits, and rats were incubated with trypsinized endotoxin. The lethal effect of endotoxin was uniformly decreased (Table I). Washed rabbit leukocytes and the "leukocytic supernate" did not increase the mortality of the recipient animals and may have reduced it slightly.

TABLE I. EFFECT OF COMPONENTS OF NORMAL BLOOD ON THE LETHAL ACTION OF TRYPSINIZED ENDOTOXIN IN THE RAT

ENDOTOXIN (ML./RAT)	COMPONENT OF BLOOD	SOURCE	VOLUME (ML./RAT)	ENDOTOXIN		ENDOTOXIN + COMPONENT†	
				D/T*	% D	D/T	% D
0.15	Serum	Human	0.5	8/10	80	5/10	50
0.15	Plasma	Human	0.5	8/10	80	3/10	30
0.25	Serum	Rabbit	1.75	9/10	90	5/10	50
0.10	Serum	Rabbit	0.5	10/16	63	4/16	25
0.06	Serum	Rabbit	1.75	3/10	30	0/10	0
0.15	Blood	Rat	0.75	13/15	87	9/19	47
0.25	Serum	Rat	0.5	12/15	80	5/15	33
0.06	Serum	Rat	0.5	4/16	25	1/15	7
0.10	Washed R.B.C.	Rat	1.0	15/15	100	7/15	47
0.15	Leukocytes	Rabbit	0.2	7/10	70	5/10	50
0.15	Leukocyte supernate	Rabbit	0.5	7/10	70	4/10	40

*D/T = Number dead/total number rats injected.
†Incubated at 25° to 27° or at 37° C. for 2 to 3 hours prior to injection.

Protection of Chick Embryos Against the Lethal Action of Endotoxin.—Tenfold serial dilutions of trypsinized endotoxin varying from 2×10^{-1} to 2×10^{-5} ml. per egg were injected into the yolk sacs of embryonated eggs, using 8 to 10 eggs per dilution. Identical concentrations of endotoxin, after incubation with 0.2 ml. of normal rabbit serum for 2 to 3 hours at 25 to 27° C. were injected into similar groups of eggs. The total volume injected per egg was 0.4 ml. Incubation with serum reduced the LD_{50} of the endotoxin for embryonated eggs from the control figure of 1.7×10^{-4} ml. to 1.3×10^{-3} ml. of endotoxin for the eggs receiving the mixture. Thus, normal rabbit serum attenuated the lethal action of endotoxin for chick embryos.

Fractionation of Protective Factor in Normal Rabbit Serum.—A preliminary attempt was made to localize the protective factor by using crude fractions of normal rabbit serum obtained by the addition of an equal volume of saturated solution of ammonium sulfate. The supernatant, labeled "albumin," and the precipitate, called "globulin," were dialyzed against running tap water and reconstituted to the original volume of the serum with neutral physiologic saline. These fractions were then incubated with trypsinized endotoxin as in the previous experiments, each animal receiving 0.5 ml. of serum fraction and sufficient endotoxin to kill 14 of 27 rats (52 per cent). The "albumin" fraction and endotoxin killed 10 of 27 rats (37 per cent), whereas the "globulin" fraction and endotoxin killed by 5 of 28 rats (18 per cent). Thus, the "globulin" fraction appeared to be more active than the "albumin" fraction in altering the toxicity of endotoxin.

TABLE II. EFFECT OF PLASMA PROTEIN FRACTIONS ON LETHAL ACTION OF ENDOTOXIN IN RATS*

PLASMA FRACTION	ENDOTOXIN (ML./RAT)	VOLUME OF PLASMA FRACTION INCUBATED WITH ENDOTOXIN, ML./RAT										
		0	1.60	1.50	1.0	0.5	0.4	0.1	0.05	0.025	0.0063	0.004
I	0.15	18/20†				14/20						
II	0.15	34/38	10/10				12/18	18/18		8/8		
	0.45	9/10				10/10						
II + III	0.15	67/73	12/20			11/34	7/20	22/37	6/9	16/27	9/9	
	0.25			4/6		3/6						
	0.50	2/2		6/6	6/6			6/6		6/6		6/6
III	0.15	26/29	6/10				13/18	12/18		14/17	7/7	
	0.25			4/6		3/6						
	0.50	2/2		6/6	5/6	5/6		5/5		6/6		6/6
III_0	0.15	18/23				21/29						
Super III_0	0.10	13/15	15/15									
IV_1	0.15	35/39				23/39		7/9	7/9	7/9		
	0.45	9/10				10/10						
IV_4	0.15	17/19				6/10		7/9	6/6	10/10		
V	0.15	8/10				9/10						

*Trypsinized end toxin incubated with plasma Fraction at 25° to 27° for 2 to 3 hours prior to injection.
†Number dead /total number injected.

Protective Action of Human Plasma Protein Fractions.—The availability of human plasma protein fractions led to investigation of the activity of such fractions in altering the lethal action of endotoxin. It is seen in Table II that

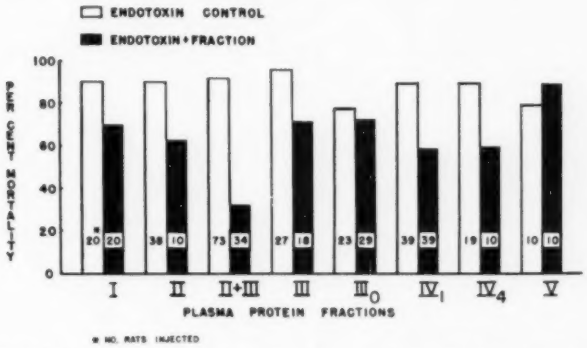

Fig. 1.—The protective effect of human plasma protein fractions. Trypsinized endotoxin was incubated at 25 to 27° C. for 3 hours with 0.4 to 0.5 ml. of the appropriate fraction.

Fractions II, II + III, III, IV_1, and IV_4 all exerted some degree of anti-endotoxic activity whereas Fractions I, III_0, Super III_0 and V were inactive or minimally active. The activities of the various plasma fractions in a volume of 0.4 to 0.5 ml. per rat are compared in Fig. 1: Fraction II + III was found to be significantly more effective in protecting against death from endotoxin than were any of the other fractions tested. Fractions III and IV_1 were also significantly protective at the 0.4 to 0.5 ml. dose level with the number of animals used. The effect of different amounts of the various fractions is illustrated in Fig. 2. Fraction II + III was effective in altering the lethal action of endotoxin in volumes as small as 0.025 ml. per rat ($P < 0.01$). The protective activity of Fraction II + III against a constant amount of endotoxin increased proportionately to the amount of the fraction used between the ranges of 0.025 and 0.8 ml. There is suggestive evidence that increasing the dose beyond 0.8 ml. led to reduction of the protective effect. Doses below 0.025 ml. were ineffective. Similar dose response curves to that obtained with Fraction II + III were obtained with varying doses of Fractions II, III, IV_1, and IV_4 incubated with trypsinized endotoxin, except that these latter fractions gave lower levels of protection generally.

Increasing the amount of endotoxin was not readily overcome by increasing the amount of active plasma protein fraction (Table II) so that the reaction is assumed not to be stoichiometric.

Effect of Varying the Type of Endotoxin.—The studies thus far reported all involved the use of trypsinized *S. typhi* endotoxin. However, lipopolysaccharides that had been prepared by the trichloroacetic acid extraction method of Boivin and associates[18] were apparently not affected by Fraction II + III (Table

III). On the other hand, attenuation of the lethal effect of endotoxin by Fraction II + III was demonstrable when the endotoxins were prepared as crude vaccines from the same organisms from which the endotoxins were derived by the trichloroacetic acid methods. The crude vaccines that were used were bacterial

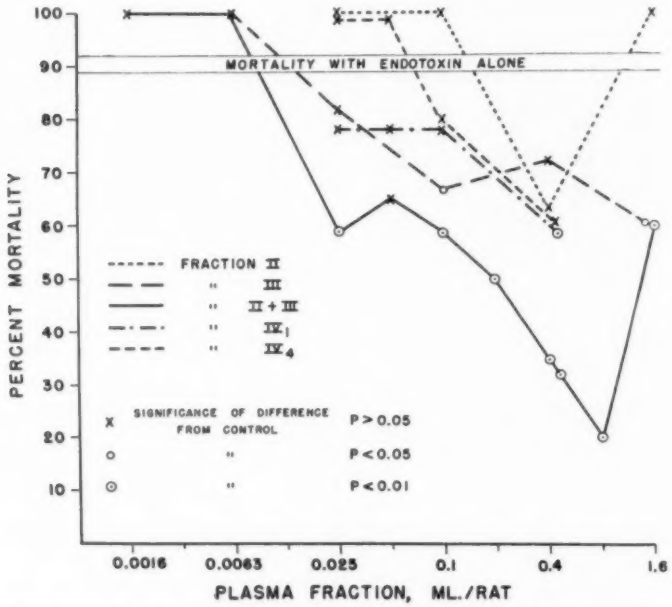

Fig. 2.—Effect of different amounts of human plasma fractions on the lethal action of endotoxin. All fractions were incubated with trypsinized endotoxin at 25 to 27° C. for 2 to 3 hours.

TABLE III. EFFECT OF FRACTION II + III ON THE LETHAL EFFECT ENDOTOXINS PREPARED BY DIFFERENT METHODS

SOURCE	METHOD OF PREPARATION	VOLUME (ML./RAT)	ENDOTOXIN		ENDOTOXIN + 0.5 ML. FR. II + III†	
			D/T*	% D	D/T	% D
S. typhimurium	$CHCl_3$ killed vaccine	1.2	6/7	86	5/10	50
Esch. coli (0127:B8)	$CHCl_3$ killed vaccine	0.8	7/15	47	0/15	0
S. typhi (0901)	$CHCl_3$ killed vaccine	1.0	5/10	50	0/10	0
S. typhi (0901)	$CHCl_3$ killed vaccine	1.5	10/15	67	0/15	0
S. typhi (Ty²)	$CHCl_3$ killed vaccine	1.0	15/20	75	3/15	20
S. typhi (Ty²)	$CHCl_3$ killed vaccine	1.5	8/10	80	0/10	0
S. typhi (Ty²)	Heat killed vaccine	1.0	9/10	90	5/10	50
S. typhi (Ty²)	Phenol killed vaccine	1.6	12/15	80	6/15	40
S. typhi (Ty²)	Formol killed vaccine	1.6	8/15	53	6/15	40
S. typhimurium	Trichloroacetic acid extract	0.6	5/10	50	5/10	50
S. typhimurium	Trichloroacetic acid extract	1.0	22/24	92	24/25	96
Esch. coli (0127:8)	Trichloroacetic acid extract	1.6	17/19	90	18/19	95
Esch. coli (0127:8)	Trichloroacetic acid extract	2.0	10/10	100	7/10	70
S. typhi (0901)	Trichloroacetic acid extract	2.0	5/9	56	2/9	22
S. typhi (0901)	Trichloroacetic acid extract	3.0	5/9	56	8/9	89

*Number dead/total number injected.
†Incubated at 25° to 27° or at 37° C. for 2 to 3 hours prior to injection.

suspensions killed by chloroform, heat, or phenol. In one experiment, formalin-killed vaccine served less well as a means for demonstrating the attenuation of the lethal effect of endotoxin by Fraction II + III (Table IV). It was concluded that differences in the manufacture of the endotoxins accounted for the differences in their response to Fraction II + III.

Properties of the Protective Factor.—Various observations on the properties of the protective factor were made during the course of these studies (Table IV). Dialyzed rabbit serum was as protective as undialyzed rabbit serum. In addition, heating rabbit serum at 56° C. for 30 minutes did not eliminate the protective activity. However, studies with Fraction II + III indicated that heating at 70° C. for one hour, or boiling, destroyed the protective activity.

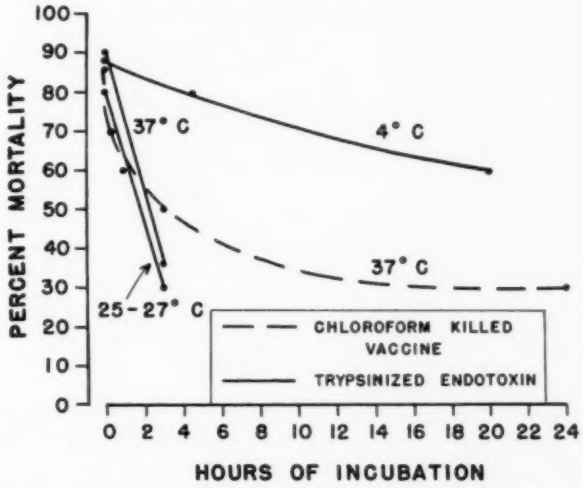

Fig. 3.—Effect of temperature and duration of incubation on attenuation of lethal action of endotoxin by Fraction II + III.

Effect of Conditions of Incubation on the Protective Action Against Endotoxin.—When 0.4 to 0.5 ml. of Fraction II + III was mixed with endotoxin immediately prior to injection, the lethal action of endotoxin was not altered significantly. Incubation of the mixture at 37° C. for one hour gave inconsistent protection. When the mixture was incubated for 3 hours either at 25 to 27° or at 37° C. consistent protection could be obtained. On the other hand, incubation of the mixture at 4 to 8° C. for 4½ hours failed to alter the toxicity of the endotoxin, but incubation for 20 hours at this temperature altered the endotoxic activity (Fig. 3).

Relationship of the Protective Factor to Properdin.—That the protective activity herein described is not properdin is suggested by several lines of evidence. First, the properdin system is inactivated in serum at 56° C. for 30 minutes,[21] whereas the protective factor against endotoxin was stable to this amount of heat. Second, Fraction II + III, which showed the most activity in attenuating

TABLE IV. PROPERTIES OF PROTECTIVE FACTOR IN SERUM AND PLASMA

SOURCE OF PROTECTIVE FACTOR	TREATMENT OF PROTECTIVE FACTOR	DOSE ML./RAT	ENDOTOXIN		ENDOTOXIN + UNTREATED PROTECTIVE FACTOR†		ENDOTOXIN + TREATED PROTECTIVE FACTOR†	
			D/T*	% D	D/T	% D	D/T	% D
Rabbit serum	56° C. for 30 min.	0.5	10/16	63	4/16	25	2/17	12
Human plasma								
Fraction II + III	56° C. for 30 min.	0.5	9/10	90	5/14	36	5/14	36
Fraction II + III	70° C. for 60 min.	0.4	9/10	90	—	—	8/10	80
Fraction II + III	100° C. for 5 min.	0.4	7/8	88	—	—	6/8	75
Fraction II + III	Repeatedly frozen to −15° C. and thawed at 37° C.	0.5	9/10	90	5/14	36	6/13	46
Rabbit serum	Dialysis	0.5	10/16	63	4/16	25	3/14	18

*D/T = Number dead/total number rats injected.
†Incubated at 25° to 27° or at 37° C. for 2 to 3 hours prior to injection.

endotoxin, is probably incomplete with respect to components of the properdin system (properdin, complements, and Mg^{++}).[21] This was inferred from the finding that Fraction II + III was not bactericidal to the strain of *S. typhi (Ty 2)* that was used. That this organism is susceptible to the action of the properdin system was suggested by the rapid killing (90 per cent in ½ hour) of the same strain by fresh rabbit serum. Third, Fraction III, from which properdin had been removed by zymosan adsorption, attenuated the lethal action of endotoxin (Table V). Fourth, solutions of pure properdin, made up to plasma concentrations, were less protective than comparable doses of Fraction II + III (Table V).

TABLE V. RELATIONSHIP OF PROPERDIN TO THE ATTENUATION OF THE LETHAL ACTION OF ENDOTOXINS BY PLASMA FRACTIONS

PLASMA FRACTION*	ENDOTOXIN		ENDOTOXIN + FRACTION	
	D/T†	% D	D/T	% D
Properdin-free Fr. III	7/15	47	2/15	13
Properdin-free Fr. III	4/10	40	0/10	0
Properdin	7/10	70	5/10	50
Fr. II + III	7/10	70	1/10	10

*All fractions given as 0.5 ml. volume per rat.
†D/T = Number dead/total number injected.

Relationship of Protective Factor to Antibody.—The possibility that the protective action of blood components is related to antibody was investigated. No precipitins and hemagglutinins against trypsinized endotoxin were found in most of the blood components used and only very low titers were found in some (Table VI). Normal rabbit serum contained no precipitins or hemagglutinins, whereas human plasma and plasma Fraction II + III contained low titers. In order to determine the role of antibody in the protective system, two types of experiments were performed. In the first, rabbits were made tolerant and immune to endotoxin and the protective action of their sera was compared with sera of unimmunized animals. In the second, antibodies were adsorbed from Fractions II + III and the adsorbed fraction was tested for its ability to attenuate the lethal action of endotoxin. Thus, normal albino rabbits (1.8 to 2.5 Kg.) were given daily pyrogenic doses of trypsinized endotoxin for 1, 2, or 7 times and their fever curves followed. The animals receiving 2 or 7 doses evidenced diminished febrile responses to successive doses of endotoxin. The precipitin and hemagglutinin levels were determined on sera obtained the day after the last injection. These sera were incubated with trypsinized endotoxin at 37° C. for 3 hours and injected into rats. Rabbit sera with no detectable precipitins and serum with a precipitin titer of 1.32 all protected against the lethal action of endotoxin.

The human plasma and plasma Fraction II + III that were used contained low titers of precipitins or hemagglutinins against trypsinized endotoxin. These antibodies were adsorbed from Fraction II + III using sensitized rabbit red cells. As can be seen in Table VI, protective activity was found in both the adsorbed and the untreated fractions. These two experiments indicate that low titers of antibody play no appreciable role in the attentuation of the lethal action of endotoxin by plasma fractions.

TABLE VI. RELATIONSHIP OF ANTIBODY TO THE ATTENUATION OF THE LETHAL ACTION OF ENDOTOXIN

COMPONENT OF BLOOD	VOLUME ML./RAT	SOURCE AND TREATMENT*	PRECIPITIN TITER	HEMAGGLUTININ TITER	ENDOTOXIN		ENDOTOXIN + COMPONENT	
					D/T	% D	D/T	% D
Serum	0.4	Rabbit, untreated	0	0	7/11	64	5/15	33
Serum	0.4	Rabbit, injected with 70 μg endotoxin I.V. once	0	0	7/11	64	6/15	40
Serum	0.4	As above, rabbits given 2 injections	0	1:8	7/11	64	7/15	47
Serum	0.4	As above, rabbits given 3 injections	1:32	1:256	7/11	64	3/15	20
Plasma	0.5	Human, untreated	1:8	—	8/10	80	3/10	30
Fr. II + III	0.4	Human, untreated	0	1:2	9/10	90	7/15	47
Fr. II + III	0.4	Human, adsorbed free of agglutinins with sensitized red cells	—	0	9/10	90	7/15	47

*Trypsinized endotoxin was used in all of these experiments.

DISCUSSION

The substance (or substances) in normal serum or plasma causing the attenuation of the lethal effect of bacterial endotoxin appears to be similar in many respects to those substances in serum or plasma that alter the chemical structure, antigenic activity, and pyrogenicity of endotoxin. Thus, the endotoxin-modifying substance described herein probably is a protein, and is found mainly in the alpha and beta globulins. Similarly, Cluff[1] found that the beta globulin fraction contained the activity causing an altered precipitation of endotoxin with homologous antibody.

The capacity of serum to attenuate the pyrogenic response as described by Goodale and co-workers[5] and by Hegemann,[6] and to split phosphorus away from endotoxin as described by Rowley,[3] were diminished by heating at 56° C. for 30 minutes. On the other hand the ability of plasma fractions to alter lethal toxicity of endotoxin was still present after such heating in the present experiments. However, no quantitation of the activity of the heated fraction was attempted. Since as little as 0.025 ml. of Fraction II + III decreased the lethal action of endotoxin, substantial reduction in the activity of an active fraction might have occurred and yet not have been detected using undiluted plasma fractions.

The protective substance herein described requires a period of incubation with endotoxin at an optimum temperature for its activity to become manifest. The same type of dependence upon time and temperature has been described by Rowley, by Hegemann, and by Goodale and associates for the respective endotoxin-modifying activities in plasma or serum that they studied. The dependence of these activities on time and temperature of incubation, the observation of the splitting away of phosphate from endotoxin upon incubation with serum,[4] and the destruction by mercuric ions of the ability of normal serum to alter the antigenicity of endotoxin[2] all suggest that an enzyme (or enzymes) in serum alters endotoxin. It may be suggested that the alteration in lethal toxicity is due also to such an enzymic action. Thus, the reduction of lethal toxicity of endotoxin by plasma or serum might be due to alteration of the endotoxin molecule, or due to the release of smaller fragments capable of blocking the lethal action.

The finding that normal whole blood alters the lethal action of endotoxin is in contrast to observations that attenuation of pyrogenicity does not occur when endotoxin is incubated with whole blood.[5] Whole blood augments the febrile response to endotoxin[8] presumably because of the release of endogenous pyrogen by leukocytes. Thus it may be inferred that endogenous pyrogen does not augment the lethal action of endotoxin. This is supported by our observation that leukocytes did not augment the lethal toxicity of endotoxin. It is also possible that the marked protective action of washed blood cells against endotoxin is in part due to the absorption of endotoxin on the red cell surface with slow elution in vivo, so that the effective amount of circulating endotoxin at any one time is diminished.

The activity of Fraction II + III in decreasing the lethal toxicity of endotoxin is noteworthy. Fraction II alone was active in a narrow range of dilution (0.1 to 1.6 ml. per rat) and it is in this range of dilution that Fraction II + III was more effective than Fraction III alone (Fig. 2). Thus, there may be an additive effect of Fractions II and III at this critical dilution. The diminished effectiveness of higher concentrations of active fractions suggests the presence of substances in plasma that inhibit the endotoxin-altering action of serum.

The protective effect of Fraction II + III was demonstrable with trypsin-treated endotoxin and with crude vaccines of organism killed by phenol, heat, or chloroform. However, the protective action was not demonstrable with endotoxin prepared by the trichloroacetic acid method,[18] which is commonly used to prepare endotoxin. The finding suggests that trichloroacetic acid in some manner alters the endotoxin molecule sufficiently to make it resistant to the attenuating action of plasma fractions. Presumably, endotoxin obtained by less drastic measures than trichloroacetic acid extraction is more closely related to the substances present in the bacterial cell and, hence, in natural infections.

The biologic significance of the presence of an enzyme that modifies endotoxin is not yet clear. Although such an enzyme might reasonably be thought to play a role in host resistance to endotoxin, direct evidence for such a role has not yet been obtained. The presence and concentration of such an enzyme may be an adaptive response to prior exposure to endotoxin-containing bacteria. Alternatively, such an enzyme in serum may represent the fortuitous circumstance that there are substances in the host that are sufficiently related chemically to endotoxin that enzymes involved in their metabolism are also able to alter endotoxin. Tissue polysaccharides with endotoxin-like properties have recently been described by Landy and Shear.[23]

SUMMARY AND CONCLUSIONS

When normal plasma or serum from man, rabbit, or rat is incubated with bacterial vaccines or with trypsinized endotoxin, the lethal action of such toxin is attenuated. Such protective activity is not demonstrable using endotoxin extracted with trichloroacetic acid.

The protective activity is found in various fractions of human plasma proteins, but is most prominent in Fraction II + III. As little as 0.025 ml. of Fraction II + III per rat may decrease the lethal action of endotoxin significantly. Very large amounts of such fractions are less active in decreasing the toxicity of endotoxin.

Homologous whole blood and washed red blood cells, also diminish the lethal action of endotoxin. Leukocytes do not increase the lethal toxicity of endotoxin.

It is suggested that the activity herein described is related to the ability of serum to alter the antigenicity, chemical structure, and pyrogenicity of endotoxin and that this activity is probably enzymic.

The assistance of Miss Phyllis Bodel, fourth year student at the Harvard Medical School, and Miss Mary I. Kendrick is gratefully acknowledged.

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LABORATORY METHODS

PROTHROMBIN MEASUREMENT BY TWO-STAGE TECHNIQUE WITH HEMOLYZED WHOLE BLOOD THROMBOPLASTIN

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PROTHROMBIN, a glycoprotein component of blood plasma, is of major importance in blood clotting. It has a probable concentration of 15 to 20 mg. per 100 ml. of normal human plasma. As complete chemical separation is not yet possible, the measurement of prothrombin is best determined by its biologic activity. This biologic activity is indirectly measured by converting the prothrombin to thrombin and then reacting the thrombin with fibrinogen. Thrombin clotting activity is directly proportional to available prothrombin and, by convention, one thrombin unit equals one prothrombin unit.

There are two major types of prothrombin activity analysis: the one-stage methods and the two-stage methods. The one-stage prothrombin determination such as the method of Quick[1] measures the summation of prothrombin and the accelerator globulins rather than prothrombin alone. Despite the lack of specificity of the one-stage test, it is still very valuable in controlling therapy with Dicumarol and similar anticoagulants, mainly because these drugs act to lower concentration of at least one of the accelerators as well as prothrombin. The one-stage methods consist essentially of adding an excess of potent tissue extract thromboplastin to plasma in the presence of an optimal concentration of calcium. The accelerated clotting time of the plasma sample is then referred to as the "prothrombin time." These "prothrombin times" can be converted to concentration or per cent of normal by constructing a reference curve based on various dilutions of normal plasmas. These curves are best constructed by diluting normal plasma with prothrombin-free plasma, but Quick[1] and also most laboratories use physiologic saline as the diluent.

The two-stage prothrombin methods such as that of Ware and Seegers[2] measure only prothrombin and are, therefore, more specific and accurate than the one-stage methods. The two-stage methods have been technically difficult to perform, and even with the present commercial availability of some of the reagents, there is a distinct lack of reproducibility of results from one laboratory to another. The two-stage methods consist essentially of adding a dilute tissue thromboplastin to defibrinated plasma in the presence of calcium as the first

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Received for publication Feb. 7, 1957.

stage, and then adding some of the generated thrombin to a fibrinogen substrate as the second stage of the test. This fibrinogen clotting time is converted into thrombin units, and the thrombin units are considered equal to the prothrombin unitage of the plasma sample.

METHODS

A simple two-stage technique for measuring prothrombin has been based on the use of hemolyzed whole blood thromboplastin.[3] The presence of a potent thromboplastic substance in red blood cells has previously been recognized by Shinowara[4] and Quick.[5] The method consists essentially of adding an aqueous calcium chloride solution to a small amount of whole blood as the first stage, and then adding an aliquot of the generating thrombin to a fibrinogen solution. The aqueous calcium chloride hemolyzes out the potent red cell thromboplastin which, together with platelets, plasma accelerators, and calcium forms a complete and extremely effective thromboplastin.

Reagents.—(1) 4.5 ml. of blood in 0.5 ml. 0.1 M sodium oxalate (buffered to pH 7.3 to 7.4 by mixing calcium chloride 2.22 Gm., 0.1 M barbital sodium 60 ml., 0.1 N HCl 40 ml., and water sufficient to make 1,000 ml.). (2) Calcium chloride, anhydrous, 0.02 M in water or sodium citrate. (3) Fibrinogen solution (prothrombin-free plasma). Prepare as follows:

> To 5.0 ml. of pooled fresh oxalated plasma add 1.0 ml. 30 per cent barium sulfate suspension (a 30 per cent suspension in either normal saline or water). Incubate 10 minutes at 37° C. with occasional mixing, centrifuge, and decant the prothrombin-free plasma.

Technique I: To 0.2 ml. oxalated or citrated blood add 9.8 ml. 0.02 M calcium chloride and incubate at 37° C. At intervals of 6, 10, and 14 minutes 0.2 ml. aliquots are added to 0.2 ml. barium plasma (fibrinogen solution) at 37° C. and the clotting time is clocked. Minimal clotting times of 13 to 30 seconds are optimal. A 15-second clotting time against oxalated barium sulfate plasma at 37° C. is equivalent to approximately 1.6 units of National Institute of Health (N.I.H.) thrombin or prothrombin (2.0 units of N.I.H. thrombin will clot 1.0 ml. of oxalated human plasma at 28° C.; only 1.6 units are necessary to clot 1.0 ml. oxalated human plasma at 37° C.). Two-tenths milliliter of oxalated blood with an hematocrit of 40 to 45 per cent represents approximately 0.1 ml. plasma. A 15-second minimal clotting time above would indicate a prothrombin unitage of 100 × 2 × 1.6 or 320 units per ml. of plasma. Three-hundred and fifty units per milliliter has been arbitrarily designated as 100 per cent. The conversion factors for minimal clotting time other than 15 seconds are given in Table I. Specimens of blood in which prothrombin concentration is low will have to be diluted with smaller volumes of calcium chloride.

A small fibrin clot forms at about 6 to 10 minutes in the generating mixture; this may be removed from the tip of the pipette with cleansing tissue if necessary. If the hematocrit varies much from the 40 to 45 per cent range, it will be necessary to proportionately correct the prothrombin concentration.

Technique II: Three-tenths milliliter of any normal blood is washed twice with normal saline to remove its plasma. Pooled washed frozen cells may be used equally well. To these washed cells add 0.12 ml. of the specimen plasma, and then add 9.68 ml. 0.02 M calcium chloride and proceed as before. A 15-second minimal clotting time would again indicate a prothrombin unitage of 100 × 2 × 1.6 or 320 units per ml. of the specimen plasma. If the specimen is serum instead of plasma, use 0.3 ml. of serum, add 0.06 ml. of fresh barium plasma (to supply antihemophilic globulin and plasma AC globulin) and then add 0.02 M calcium chloride in an amount sufficient to bring total volume to 10.0 ml. If the specimen plasma is more than two days old, it is best to insert 0.06 ml. fresh barium plasma before adding enough 0.02 M calcium chloride to make 10.0 ml. If the specimen plasma is completely deficient in serum AC globulin, then it is best to insert 0.1 ml. of a normal serum diluted $\frac{1}{10}$ with normal saline.

TABLE I. CONVERSION TABLE FOR TWO-STAGE METHOD

CLOTTING TIME (MINIMAL) SECONDS (37° C.)	FACTOR (MULTIPLY TIMES TOTAL PLASMA OR SERUM DILUTION TO GIVE PROTHROMBIN UNITS PER ML.)
11	4.00
11.5	3.60
12	3.20
12.5	2.90
13	2.55
13.5	2.25
14	1.90
14.5	1.75
15	1.60
15.5	1.50
16	1.45
16.5	1.35
17	1.30
18	1.20
19	1.10
20	1.05
21	0.95
22	0.90
23	0.80
24	0.70
25	0.65
26	0.60
27	0.58
28	0.55
29	0.53
30	0.51
31	0.50
32	0.48

Three hundred and fifty units = 100 per cent.

Fifteen-second clotting time against oxalated plasma at 37° C. is equivalent to 1.6 N.I.H. units (National Institute of Health) of prothrombin or thrombin.

RESULTS

The prothrombin content of normal human plasma is about 350 units per ml. by this method. These are equivalent to N.I.H. units of prothrombin or thrombin. The N.I.H. unit of thrombin is that amount which will clot 1 c.c. of a standardized solution of fibrinogen in 15 seconds at 28° C.; 2 units of N.I.H. thrombin will clot 1.0 ml. of oxalated human plasma in 15 seconds at 28° C. The method of Ware and Seegers[2] yields about 325 units of prothrombin per ml. of human plasma. The TAMe assay method of Glueck and associates[6] yields about 54 units of prothrombin per milliliter of plasma.

There is an obvious question raised. Which is the correct value—350, 325, or 54 units per ml. of plasma? An attempt is made in all the two-stage methods to capture and measure all of a labile biologic activity. The higher the yield of thrombin or prothrombin units, the closer one should be to the absolute figure. At some future time the amount of prothrombin in plasma will be determined accurately in terms of weight rather than loosely in terms of activity. For the present, the two-stage method by any good technique must be expressed as a per cent of normal as well as in units.

The red cell thromboplastin, on which the described two-stage method depends, is a fascinating material. It is a partial thromboplastin functioning only in the presence of platelets and other thromboplastin precursors. These other

precursors, together with platelets, can make a thromboplastin of moderate potency without help from hemolyzed red cell material. The addition of this red cell material causes about a fourfold increase in potency. This can be demonstrated by adding to normal blood first an isotonic solution of calcium chloride in saline (0.02 M $CaCl_2$ in 0.53 per cent saline solution) which does not hemolyze the red cell, and then doing the same with the hemolyzing solution of 0.02 M $CaCl_2$ in water.

In the two-stage prothrombin test, then, whole blood thromboplastin is more potent than tissue extracts. It is hard to compare the potencies in the one-stage tests because of the difficulty of separating thrombin from the generating whole blood thromboplastin, but the tissue extracts seem to be more powerful in the one-stage reactions. The natural thromboplastin generated by platelets plus serum and prothrombin-free plasma and calcium in the Biggs-MacFarlane[8] thromboplastin generation test is quite potent, but seems to be partially mixed with thrombin. Clinical significance of red cell thromboplastin is not apparent yet, but it is conceivable that thrombotic phenomena of hemolytic anemias and polycythemia may be related to this factor. In 100 determinations on normal individuals by this test the average prothrombin concentration was 362 units per milliliter plasma. The median was 350 units ± 30. The level of 350 units per ml. was, therefore, conveniently selected to represent 100 per cent concentration of prothrombin.

TABLE II. PROTHROMBIN VALUES BY ONE- AND TWO-STAGE METHODS

ONE-STAGE METHOD (SECONDS)	ONE-STAGE METHOD (PER CENT) SALINE DILUTION OF QUICK	ONE-STAGE METHOD (PER CENT) BARIUM SULFATE PLASMA DILUTION OF ROSENFELD AND TUFTS	TWO-STAGE METHOD (PER CENT)
12*	100*	100*	100*
15	60	25	30
18	45	20	21
20	38	15	18
24	25	10	12
30	20	7	8
40	10	4	4

*Normal people, not on anticoagulants.

TABLE III. COMPARISON OF BLOOD THROMBOPLASTIN TWO-STAGE METHOD WITH SEEGERS TWO-STAGE METHOD

SPECIMEN PLASMA NO.	ONE-STAGE PROTHROMBIN TIME (SECONDS)	TWO-STAGE PROTHROMBIN BLOOD THROMBOPLASTIN METHOD (DRESKIN) (PER CENT)	TWO-STAGE PROTHROMBIN* WARE AND SEEGERS[2] (PER CENT)
1	13	91	86
2	13.5	100	90
3	13.5	100	90
4	14	72	90
5	16	60	78
6	20	23	33
7	22	23	16
8	24	40	45
9	27	11	12
10	34	8	13

*Commercial Difco Thromboplastin and AC globulin with pooled human barium plasma as the fibrinogen. One hundred units of prothrombin per milliliter of plasma was found equivalent to 100 per cent.

A comparison has been made of joint prothrombin determinations by the one-stage Quick method and the two-stage method in a group of ambulatory patients taking Dicumarol or Hedulin (Table II). Percentages by the one-stage method are best compared on the basis of barium sulfate plasma dilution (Rosenfeld and Tufts[7]) rather than on the saline dilution of Quick.[1] A further comparison of two-stage prothrombin value obtained by the blood thromboplastin technique with values measured by the classical technique of Ware and Seegers[2] showed good correspondence (Table III).

SUMMARY

A new two-stage method of measuring prothrombin activity is described. The method is based on the use of a natural thromboplastin derived from hemolyzed red blood cells. A high yield of prothrombin is obtained and measured by a relatively simple method employing a minimum of reagents.

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USE OF A NONIONIC DETERGENT IN PAPER ELECTROPHORESIS OF SERUM PROTEINS

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INTRODUCTION

THE development of simple systems for the fractionation of serum proteins by paper electrophoresis has resulted in widespread adaptation of this technique as a clinical tool. However, the results obtained using paper electrophoresis have corresponded neither to those obtained using moving boundary electrophoresis nor to those obtained using the routine salt fractionation procedures which have been employed for so many years. For example, Jencks, Smith, and Durrum,[1] using paper electrophoresis, obtained a mean value of 68.9 per cent albumin (albumin-globulin ratio 2.2) from sera of 185 normal individuals, while Armstrong, Budka, and Morrison,[2] using moving boundary electrophoresis, obtained a value of 59 per cent albumin† (albumin-globulin ratio 1.4) from pooled human serum. Salt fractionation procedures normally yield albumin-globulin ratios of 1.1 to 2.0.[3, 4]

A modification of one of the most commonly used paper electrophoretic procedures has been developed in this laboratory which yields results comparable to those obtained from moving boundary electrophoretic or salt fractionation procedures.

METHODS AND MATERIALS

Durrum's method[5] for the electrophoretic separation of human serum proteins was modified only by the addition of varying amounts of Sterox SE‡ to the barbital-sodium barbital buffer (pH 8.6, ionic strength 0.075) which was used in all cases. Runs were conducted on Spinco§ equipment for 16 hours at 80 volts. The addition of Sterox did not affect the pH of the buffer. The protein patterns, dyed with bromphenol blue, were analyzed using the Spinco Analytrol densitometer.

RESULTS AND DISCUSSION

A comparison of the relative per cent of serum albumin determined by sodium sulfite fractionation[6] to that determined by paper electrophoresis using

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Received for publication May 31, 1957.
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†The above value was obtained by adjusting the 55 per cent albumin value reported for pooled plasma to correct for the fibrinogen content.
‡A polyoxyethylene thioether available from Monsanto Chemical Co., St. Louis, Mo.
§Spinco Division, Beckman Instruments, Inc., Belmont, Calif.

varying amounts of Sterox in the buffer is shown in Table I. Correspondence between the two methods is improved for all concentrations of Sterox used.

TABLE I. MEAN ALBUMIN VALUES

STEROX (PER CENT)	ALBUMIN (BIURET) (PER CENT)	ALBUMIN (ELECTROPHORESIS) (PER CENT)	NUMBER OF SPECIMENS
0.00	54.3	48.4	22
0.02	54.3	54.0	13*
0.20	58.7	57.7	15
1.00	54.3	55.5	13*

*Same specimens.

When 0.2 per cent by volume of Sterox is added to the buffer, greatly increased resolution of the protein pattern is observed (Fig. 1). Lloyd and Stewart[7] have recently reported similar results using another nonionic detergent, Lubrol W.

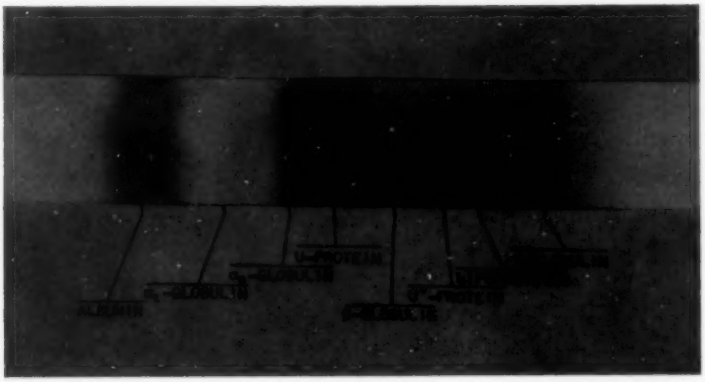

Fig. 1.—Electrophoretic pattern of serum from patient suffering from glomerular nephritis, using 0.20 per cent Sterox in the buffer.

While increasing concentrations of Sterox yield increasing resolution of the protein pattern, it is impractical to use high concentrations of Sterox because of the inordinate amount of foam these solutions produce. For this reason, buffer containing 0.2 per cent Sterox was chosen for use in later runs. The relative proportions of the alpha-1 globulins decrease significantly as Sterox concentration is increased. However, this is thought to be a result of decreased albumin tailing.

Using 0.2 per cent Sterox, the following effects have been observed. A well-defined protein band appears between the alpha-2 and beta globulins. This band appears to be identical to that found by Stern,[8] and is called U-protein. The observed migration ratio* is 0.49. Another well-defined band having a migration ratio of 0.76 appears between the beta and gamma globulins. This band is tentatively called U′-Protein. Finally, a sharp band, comprising about

*Defined[8] as the distance between the band in question and the albumin band divided by the distance between the albumin band and the globulin band.

5 per cent of the total protein, appears among the gamma globulins. It has been found that its migration ratio varies inversely with the concentration of Sterox in the buffer. Sudan IV, a lipid dye, has been used to stain this fraction; in one instance, using this stain, a resolution into two distinct fractions was observed. This last band also is stained by bromphenol blue; therefore, it may be composed of beta lipoproteins. A fast moving lipid bearing alpha-globulins found in electrophoretically separated albumin at pH 8.6 may also be included in these bands.

Serum was obtained from 49 normal individuals who were undergoing routine serologic examinations in connection with military enlistments or required annual physical examination. These sera were treated in the manner described. The results are shown in Table II. It can be seen that the albumin-globulin ratios correspond well to the values obtained using salt fractionation, and that, in general, the mean value for each protein fraction shows improved correspondence with results obtained using moving boundary electrophoresis.

TABLE II. NORMAL PROTEIN VALUES

COMPONENT	MEAN VALUE (PER CENT)	STANDARD DEVIATION (PER CENT)	NORMAL RANGE MEAN ± 2 S.D. (PER CENT)	MOVING BOUNDARY ELECTROPHORESIS (PER CENT)
Albumin	59.9*	5.1	49.7-70.1†	59.0
α_1-Globulin	2.6	0.5	1.6- 3.6	5.6
α_2-Globulin	7.0	2.1	4.9- 9.1	9.3
β-Globulin*‡	11.9	2.5	6.9-16.9	14.3
γ-Globulin§	13.5	3.1	7.3-19.7	11.8
Lipoprotein	5.1	1.7	1.7- 8.5	-

*Albumin-globulin ratio 1.49.
†Albumin-globulin ratio range 0.99-2.34.
‡Includes U-protein.
§Includes U′-protein.

At present, over one hundred specimens have been analyzed using 0.2 per cent Sterox. U-protein and U′ protein have been observed in about 80 per cent of these specimens. With the exception of a few cases they appear in small relative percentages. The resolution of U and U′ bands is valuable primarily with respect to their potential clinical utility. In view of the heterogeneity of other electrophoretically separated serum fractions, it is expected that these also will be mixtures. Furthermore, there remains a possibility that multilayer adsorptions of the type postulated by Putnam[9] to describe his investigations in the albumin-dodecyl sulfate system may account for these bands.

SUMMARY

Correspondence between albumin values obtained using salt fractionation procedures or moving boundary electrophoresis and paper electrophoresis is improved by the inclusion of Sterox SE, a nonionic detergent, in the electrophoresis buffer.

Using 0.2 per cent Sterox three well-defined bands are observed in addition to the ones commonly found. One of these has been shown to be a lipoprotein.

Values for serum proteins of 49 normal individuals were obtained using 0.2 per cent Sterox in the electrophoresis buffer.

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SIMPLIFIED COLORIMETRIC METHOD FOR TRUE BLOOD GLUCOSE

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INTRODUCTION

THE majority of methods for the determination of blood glucose are based upon the ability of glucose in hot alkaline solution to reduce certain metallic ions of which the cupric and ferricyanide ions are most commonly used. The extent of reduction is then established by colorimetric, titrimetric, or gasometric methods.

It has long been known that there are reducing substances other than glucose present in the blood and that these may occur in sufficient amounts to increase considerably the "apparent" glucose values.

The various blood sugar reduction methods differ in their specificity for glucose and, therefore, may give slightly or even significantly different values for both normal and pathologic blood.

Nelson[1] and Somogyi[3] have approximated true glucose values by preparing a blood filtrate containing practically no reducing substances other than glucose; however, their copper method is not stoichiometric and also requires several steps and reagents.

Folin and Wu[4] introduced the tungstic acid filtrate and their method has found wide application in hospital laboratories for it lends itself to the determination of many other materials as well as glucose; unfortunately, Folin and Wu's copper procedure yields glucose values with positive errors of 20 mg. per cent or more due to the presence of reducing substances other than glucose in the filtrate.

By using a modified reagent introduced by Timell and co-workers[5] a procedure has been developed for determining true glucose values using either the tungstic acid filtrate of Folin and Wu or the barium hydroxide, zinc sulfate filtrate of Nelson and Somogyi. In addition to the obvious advantage of dealing with true glucose levels, the method is technically easy to perform and uses only a single reagent which is stable over a long period of time.

MATERIALS AND METHODS

Reagents.—(1, A) 2-aminobiphenyl, melting point 50 to 52° C.† The recrystallization of this material is not necessary. (1, B) Analytical grade glacial acetic acid. Exactly 8.0 Gm. of the amine is dissolved in two liters of glacial acetic acid, or 8.6 Gm. of the amine in a 5-pound bottle of glacial acetic acid (0.4 per cent solution of aminobiphenyl in glacial acetic

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Received for publication June 28, 1957.
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†Obtained from Eastman Kodak Company, Rochester, N. Y.

acid) to form the single reagent used in this procedure. This is allowed to stand for at least 2 to 3 days in the dark before use. By this time a slight yellow coloration will have formed and will remain quite constant for at least a month when kept in the dark. (2) Merck's anhydrous glucose in 0.2 per cent benzoic acid was used for the preparation of standard glucose solutions.

Apparatus.—The reaction is carried out in open test tubes (150 × 16 mm.). The use of glass-stoppered tubes results in only slightly better reproducibility.

A Coleman Junior spectrophotometer is used with 19 × 105 mm. cuvettes to measure the transmittance.

Procedure.—Prepare either (a) Folin and Wu's tungstic acid filtrate or (b) Somogyi's barium hydroxide and zinc sulfate filtrate so that there is a final blood dilution of 1:10.

One milliliter of either filtrate (the concentration of glucose should be within the range of 0.01 to 0.5 mg./ml.) is transferred with a calibrated pipette to a test tube and 5 ml. of the reagent (a 0.4 per cent solution of 2-aminobiphenyl in glacial acetic acid) is added with an automatic burette.

The test tube is inserted in a boiling water bath and heated for 45 minutes (analyses were usually carried out in duplicate). A reagent blank is also included (1 ml. H_2O + 5 ml. of the reagent). After completion of the reaction, the tubes are cooled in water and the transmittance is measured at a wave length of 650 mμ against the reagent blank set at 100 per cent transmittance. From the average values thus obtained and the known relationship between transmittance and concentration the amount of glucose present is immediately obtained.

Calibration.—A calibration curve is established by accurately preparing several different aqueous solutions of glucose ranging in strength from 10 to 500 mg. per 100 ml. These standard solutions must be further diluted with distilled water to 1:10 (1 ml. standard diluted to 10 ml. with distilled water). One milliliter of each of these dilute standard solutions is then processed as 1 ml. of blood filtrate and the transmittance plotted on semilogarithmic paper.

RESULTS AND DISCUSSION

This aromatic amine condenses with the glucose forming a characteristic green color when heated in glacial acetic acid to which has been added a deproteinized blood filtrate. The time of heating necessary to produce the maximum transmittance is 45 minutes with concentrations varying from 10 to 500 mg. per cent glucose. There is no change in transmittance when heated beyond this period.

After removal from the heating bath the transmittance remained constant for the first 2 hours, but after 24 hours, slightly lower values were noted (Table I).

TABLE I. CHANGE IN PER CENT TRANSMITTANCE WITH TIME

GLUCOSE MG. PER CENT	PER CENT TRANSMITTANCE AT 650 mμ			
	A	B	C	D
25	89	90	89	90
50	80	81	80	81
100	65	64	65	66
150	52	52	52	54
200	42	42	42	44
250	34	34	34	35
300	27	28	27	28
400	20	19	20	21

A and B are duplicate experiments; C are values found for A after 2 hours; D are values found for B after 24 hours.

The acetic acid solution of the reagent when freshly prepared is colorless but turns slightly yellow 24 to 48 hours after preparation. Thereafter it remains quite stable for at least a month and probably much longer, when kept in the dark. Determinations run at different times with the same reagent failed to demonstrate any discrepancy in values with fresh standard glucose solutions (Table II).

TABLE II

GLUCOSE (MG. PER CENT)	FRESHLY PREPARED REAGENT (% TRANS.)	3-DAY-OLD REAGENT (% TRANS.)	15-DAY-OLD REAGENT (% TRANS.)	30-DAY-OLD REAGENT (% TRANS.)
50	82	80	80	81
100	67	64	63	64
150	56	52	51	52
200	45	42	42	41
250	36	34	34	35
300	30	27	28	28
400	22	19	19	20

TABLE III. GLUCOSE RECOVERIES FROM FOLIN-WU BLOOD FILTRATES (MG. PER CENT)

ADDED GLUCOSE (MG. %)	A*	B	C	D	E	F	AVERAGE PER CENT RECOVERY
0	85	77	83	77	88	83	
25	109	101	110	105	113	108	102.0
50	133	130	133	130	138	133	101.3
100	183	178	183	177	188	184	100.0

*A through F represent different protein-free filtrates.

TABLE IV. COMPARISON OF METHODS

FOLIN-WU Cu METHOD (MG. % GLUCOSE)	NELSON-SOMOGYI Cu METHOD (MG. % GLUCOSE)	GLYCOSYLAMINES	
		FOLIN-WU FILTRATE (MG. % GLUCOSE)	SOMOGYI FILTRATE (MG. % GLUCOSE)
89	65	68	68
400	500+	500+	500+
308	271	268	275
97	65	68	63
91	72	70	70
195	150	145	145
130	97	95	100
123	122	120	120
108	95	88	92
190	170	168	168
97	75	75	75
123	110	110	110

SUMMARY

The condensation of glucose with 2-aminobiphenyl resulting in the formation of a characteristic color, the intensity of which is directly proportional to the quantity of glucose, has been adapted to a simple technical procedure using a single stable reagent. Values obtained by this procedure which may be used with either Folin-Wu or Somogyi-Nelson deproteinized filtrates reflect true blood glucose.

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SEPARATION OF BETA LIPOPROTEIN FROM NORMAL AND HYPERLIPEMIC HUMAN SERA

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ADDITION of heparin and phenol to serum induces turbidity which is cleared by centrifugation.[1] The ratio of cholesterol to phospholipid in the precipitate is similar to that of beta lipoproteins separated by ultracentrifugation[2] or chemical fractionation.[3] This seemed to provide a simple method for measuring beta lipoprotein. The purpose of our investigation was to test this hypothesis by comparing the beta lipoprotein fraction obtained by ultracentrifugation and by heparin-phenol precipitation.

METHODS AND RESULTS

Blood was drawn from fasting human beings and the serum was separated immediately after clotting; care was taken to avoid hemolysis. The sera were stored at +4° C. from 24 hours to two weeks before analysis. A solution of 5 per cent phenol in 0.85 per cent sodium chloride was freshly prepared before use because inconsistent results were obtained with old phenol solutions. Heparin containing 1,000 U.S.P. units per ml. was used.

Beta lipoproteins were separated ultracentrifugally from sera at salt density of 1.063 by the method of Gofman and co-workers.[4] Cholesterol and phospholipids were extracted with chloroform-methanol 1:1 as described by Folch and associates.[5] Extractions with alcohol-ether 3:1 were also performed in some samples. Since the results were similar, the Folch method was generally used. No significant difference in results was found when the extraction procedure was applied to samples before or after removal of the diffusible substances by dialysis.

Cholesterol was determined by the method of Abell and associates.[6] Phospholipid values were calculated by multiplying lipid phosphorus as determined by the method of Fiske and SubbaRow[7] by 25.

A. *Interactions of Serum Lipoproteins With Heparin and Phenol.*—

Effect of salt concentrations: Each tube contained 1 ml. of serum diluted with 9 ml. of sodium chloride solutions of varying concentrations from 0.85 per cent to 10 per cent. Heparin (100 U.S.P. units in 0.1 ml.) was added and then 2 ml. of 5 per cent phenol in 0.85 sodium chloride solution. The precipitate was separated by centrifugation after an incubation period of 24 hours. Quantities of cholesterol and phospholipids in the precipitate decreased in inverse proportion to the increase of salt concentration. C/P ratios were found to have constant values, however. At salt concentrations around 0.85 and 1.0 per cent the values of cholesterol, phospholipids, and the C/P ratios were similar to those found in beta lipoproteins separated by the ultracentrifuge.

Effect of phenol and heparin concentrations: To a system containing 1 ml. of serum, 9 ml. of 0.85 per cent sodium chloride, 100 U.S.P. units of heparin, and 2 ml. of phenol

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Supported by Grant No. H2201, Department of Health, Education, and Welfare, U. S. Public Health Service.

Received for publication July 19, 1957.

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solutions from 0.5 to 5 per cent in 0.85 per cent sodium chloride were added. Higher concentrations were not used because of the insolubility of phenol. Cholesterol and phospholipids in the precipitate, separated by centrifugation after 24-hour incubation, increased proportionally with increase of phenol concentration. When a phenol concentration of 5 per cent was used, the cholesterol, phospholipids, and C/P ratios were similar to those obtained in beta lipoproteins isolated by ultracentrifuge. At low concentration of phenol incomplete precipitation of cholesterol and phospholipids occurred.

Results in Table I show no difference in amount of lipoprotein precipitated using different concentrations of heparin (from 100 to 2,000 U.S.P. units). Values for cholesterol, phospholipids, and C/P ratio in the precipitate were similar to those obtained from beta lipoproteins isolated by ultracentrifugation.

TABLE I. INTERACTIONS OF SERUM LIPOPROTEINS WITH HEPARIN AND PHENOL EFFECT OF HEPARIN CONCENTRATION ON C/P RATIO

HEPARIN CONCENTRATION IN U.S.P. UNITS	PRECIPITATE OBTAINED FROM SERUM AFTER ADDITION OF HEPARIN AND PHENOL*		
	CHOLESTEROL (MG./1 ML.)	PHOSPHOLIPIDS (MG./1 ML.)	C/P
2,000	1.23	0.92	1.33
1,000	1.28	0.93	1.37
500	1.26	0.92	1.36
200	1.23	0.94	1.30
100	1.28	0.95	1.34
Lipoproteins separated by ultracentrifugation D. 1.063	1.25	0.93	1.34

*Each tube contained: Serum, 1 ml.; 0.85 per cent sodium chloride, 9 ml.; heparin, concentrations indicated above; and 5 per cent phenol, 2 ml. Incubation time: 24 hours.

TABLE II. INTERACTIONS OF SERUM LIPOPROTEINS WITH HEPARIN AND PHENOL; EFFECT OF INCUBATION TIME ON C/P RATIO

INCUBATION TIME (HOURS)	PRECIPITATE OBTAINED FROM SERUM AFTER ADDITION OF HEPARIN AND PHENOL*		
	CHOLESTEROL (MG./1 ML.)	PHOSPHOLIPIDS (MG./1 ML.)	C/P
1	0.95	0.57	1.66
2	0.94	0.57	1.64
5	1.11	0.67	1.65
18	1.24	0.76	1.63
24	1.24	0.75	1.64
48	1.24	0.76	1.63
Lipoproteins separated by ultracentrifugation at D. 1.063	1.24	0.76	1.63

*Each tube contained: Serum, 1 ml.; 0.85 sodium chloride, 9 ml.; heparin, 100 U.S.P. units; 5 per cent phenol in 0.85 per cent sodium chloride, 2 ml.

Effect of incubation time: The amounts of cholesterol and phospholipids precipitated increased proportionally with prolongation of incubation up to 18 hours (Table II) when the values were similar to those obtained in beta lipoproteins separated by ultracentrifugation. Prolonged periods of incubation (48 hours) did not alter the values. For each incubation period C/P ratios were similar.

From these results it appears that the precipitate obtained from sera after addition of heparin and phenol and the beta lipoprotein fraction separated by ultracentrifugation at salt density 1.063 are similar. This suggests that beta lipoproteins may be measured in human sera by a simple technique which can be carried out in any clinical laboratory.

To test this method, comparative studies were made on lipoproteins separated by ultracentrifugation and by the heparin-phenol method from normal and hyperlipemic sera. Cholesterol to phospholipid ratios and total cholesterol content were determined in each sample.

B. *Clinical Application.*—

Heparin-phenol precipitation technique: One milliliter of serum was diluted with 9 ml. of 0.85 per cent sodium chloride; 100 U.S.P. units of heparin was added and the tube was gently shaken; finally, 2 ml. of 5 per cent phenol in sodium chloride 0.85 per cent was added, the solution mixed by inversion, and allowed to remain at room temperature (approximately +24° C.) for 24 hours. At the end of this incubation period, the tube was centrifuged at 3,000 r.p.m. for 30 min. and the precipitate obtained by decantation. The precipitate was resuspended in 1 ml. of saline and extracted as described above.

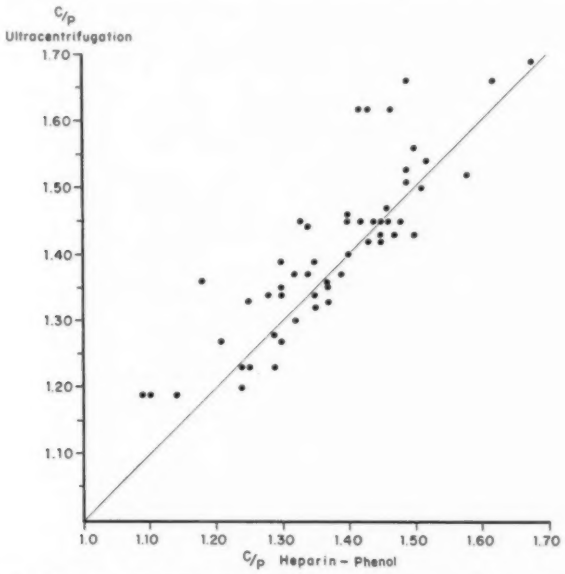

Fig. 1.—Correlation of C/P ratios in beta lipoproteins separated by ultracentrifugation and heparin-phenol precipitation ($r = 0.86 \pm 0.02$).

RESULTS

Sera from 60 normal and abnormal patients were studied. In each specimen total lipid and cholesterol values and lipoprotein pattern had been determined previously. Sera from abnormal subjects were divided into 2 classes: (1) normolipemic, normocholesterolemic with relatively high levels of alpha lipoproteins; (2) hyperlipemic, normo-, or hypercholesterolemic with high levels of very low-density lipoproteins. In some samples of this latter group with total lipid values ranging from 3,000 to 4,000 mg. per cent, results were not satisfactory when undiluted serum was used. Preliminary dilution was necessary to a total serum lipid level of 600 to 900 mg. per cent before addition of heparin and phenol. In 3 of these sera, even after dilution, addition of heparin and

TABLE III. C/P RATIOS IN BETA LIPOPROTEINS SEPARATED BY ULTRACENTRIFUGATION AND BY HEPARIN-PHENOL PRECIPITATION IN SERA BEFORE AND AFTER REMOVAL OF LOW-DENSITY LIPOPROTEINS*

	SERUM						BETA LIPOPROTEINS FROM TOTAL SERUM				BETA LIPOPROTEINS FROM SERUM AFTER REMOVAL OF VERY LOW-DENSITY LIPOPROTEINS			
			ULTRACENTRIFUGAL PATTERN (D 1.24 KBr-NaCl)†				CHOLESTEROL		C/P		CHOLESTEROL		C/P	
NO.	TOTAL LIPID (MG. %)	TOTAL CHOLESTEROL −S (MG. %)	−S 70–400 (MG. %)	40–70 (MG. %)	25–40 (MG. %)	1–10 (MG. %)	HEPARIN + PHENOL (MG. %)	ULTRAC.‡ (MG. %)	HEPARIN + PHENOL	ULTRAC.	HEPARIN + PHENOL (MG. %)	ULTRAC. (MG. %)	HEPARIN + PHENOL	ULTRAC.
1	700	191	0	26	235	220	106	113	1.32	1.34	108	110	1.34	1.33
2	650	210	35	40	430	181	121	131	1.28	1.30	124	130	1.28	1.32
3		288	47	59	400	203	135	191	1.34	1.36	138	205	1.32	1.34
4		200	42	75	350	160	128	138	1.30	1.32	134	140	1.28	1.30
5	3,450	654	1,400	104	77	<30	34	324	1.38	1.75	42	39	1.40	1.40
6	1,585	319	295	104	118	60	45	208	1.45	1.65	38	40	1.43	1.45
7	2,163	250	480	47	128	40	44	150	1.30	1.60	48	52	1.23	1.30

*Low-density lipoproteins were separated by spinning the sera at 30,000 r.p.m. for 2 hours.
†Normal values in mg./100 ml. (men 35 to 60 years old): −S 70–400:60 ± 10; −S 40–70:33 ± 1; −S 25–40:285 ± 17; −S 1–10:180 ± 5.
‡Concentrated at D. 1.063.

J. Lab. & Clin. Med.
February, 1958

phenol did not produce a sediment after 24-hour incubation and centrifugation; a yellow top layer was observed which could be separated from the subnatant by filtration, using Whatman filter paper No. 1. The top layer had a C/P ratio similar to that of the usual beta lipoprotein precipitate.

In Fig. 1, C/P values of the precipitate are plotted against C/P ratios obtained from beta lipoproteins separated by ultracentrifugation. The good correlation of values was otherwise confirmed by calculation of the correlation factor, $r = 0.86 \pm 0.2$.

In some cases C/P values for lipoproteins isolated by the two methods did not correspond. After addition of heparin and phenol, the supernatant was not clear after separation of the precipitate by centrifugation. This phenomenon may have been due to the presence of some low-density lipoproteins not precipitable by heparin and phenol. This explanation was supported by the fact that the lipoprotein pattern obtained by ultracentrifugation showed high levels of low-density lipoproteins (Table III; sera 5, 6, and 7). Prior removal of very low-density lipoproteins in these particular sera by spinning at 30,000 r.p.m. for 2 hours abolished the differences observed in C/P ratios in the beta lipoproteins separated by the two methods. Normal (1, 2, 3, and 4) and pathologic sera with high levels of low-density lipoproteins (5, 6, and 7) were studied before and after removal of the chylomicron fractions. In both, beta lipoproteins were separated by ultracentrifugation and by heparin-phenol precipitation. In sera 1, 2, 3, and 4, no differences were observed before and after removal of chylomicrons. Before removal of the chylomicrons. Before removal of the chylomicron fraction sera 5, 6, and 7 showed higher values for the C/P ratio in the beta lipoproteins separated by ultracentrifugation (1.75, 1.65, and 1.60) compared with those from heparin-phenol precipitation (1.38, 1.45, and 1.30). After removal of chylomicrons, no difference in C/P ratios was observed by the two methods. The values were similar to those observed in the heparin-phenol precipitate before the removal of the chylomicrons.

Cholesterol content of beta lipoprotein fractions was separated by ultracentrifugation and by heparin-phenol precipitation: lipoproteins were isolated by ultracentrifugation at salt density 1.063. The bottom material, after removal of top layer from ultracentrifuge tube, was concentrated by pervaporation to 1 ml. To aliquots of the same sera, heparin and phenol were added; after 24 hours the precipitate (beta lipoprotein) and supernatant were separated. Supernatant was concentrated to 1 ml. by pervaporation. Cholesterol content was determined in these 4 fractions. Data on 26 sera are presented in Table IV. The sum of cholesterol values of top and bottom fractions (ultracentrifuge) and the sum of cholesterol values of supernatant and precipitate (heparin-phenol) was very similar to the cholesterol values determined in the original serum. Good correlation was also observed between cholesterol content of top fractions (ultracentrifuge) and precipitate (heparin-phenol) (Fig. 2). The calculated correlation factor was $r = 0.98 \pm 0.02$.

In some hyperlipemic sera (5, 6, and 7; Table III) satisfactory correspondence was obtained only after removal of very low-density lipoproteins and dilution of the sera to values of total lipids around 600 to 900 mg. per cent.

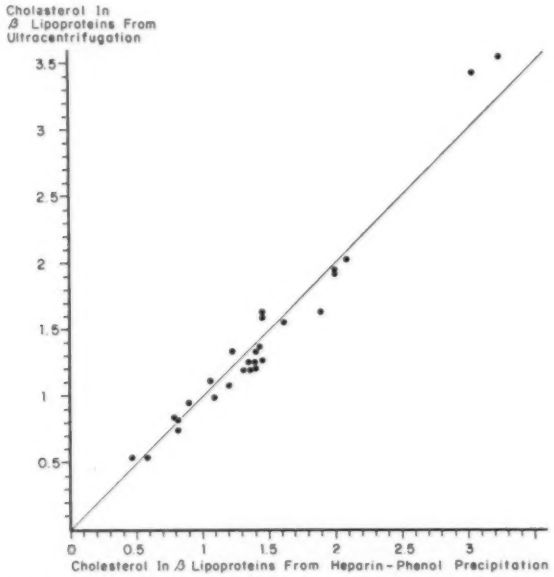

Fig. 2.—Correlation of cholesterol content of beta lipoproteins separated by ultracentrifugation and heparin-phenol precipitation ($r = 0.98 \pm 0.02$).

DISCUSSION

It appears that addition of appropriate amounts of heparin and phenol to normal serum induces formation of a precipitate with a cholesterol/phospholipid ratio and cholesterol content similar to that of beta lipoproteins separated by ultracentrifugation at salt density 1.063. The values are similar to those reported for beta lipoproteins separated by chemical fractionation.[3] The quantitative separation of serum beta lipoproteins seems achieved by heparin-phenol precipitation.

In some sera with high levels of total lipids ($>$ 1,200 mg. per cent) and low-density lipoproteins (–S 70–400$>$100 mg. per cent and –S 40–70$>$75 mg. per cent) the method had limitations; preliminary dilution to a total serum lipid concentration of between 600 to 900 mg. per cent was required for the method to be successful. Therefore, in hyperlipemic sera, determination of total lipids before application of this method is necessary. Further, beta lipoproteins separated from these sera by the precipitation method may show variations in cholesterol content and the ratio of cholesterol to phospholipid from lipoprotein fractions separated by ultracentrifugation at D. 1.063. This can be explained by the fact that in these particular sera some cholesterol is

TABLE IV. CHOLESTEROL VALUES IN SERUM AND LIPOPROTEIN FRACTIONS SEPARATED BY ULTRACENTRIFUGATION D. 1.063 (TOP AND BOTTOM) AND HEPARIN-PHENOL PRECIPITATION (PRECIPITATE AND SUPERNATANT)

SERUM N.	SERUM (MG./1 ML.)	LIPOPROTEIN FRACTIONS (ULTRACENTRIFUGATION) TOP (MG./1 ML.)	BOTTOM (MG./1 ML.)	TOTAL (MG./1 ML.)	LIPOPROTEIN FRACTIONS (HEPARIN + PHENOL) PRECIPITATE (MG./1 ML.)	SUPERNATANT (MG./1 ML.)	TOTAL (MG./1 ML.)
A	1.80	1.21	0.42	*1.63*	1.32	0.37	*1.69*
B	4.43	3.44	0.62	*4.06*	3.06	0.67	*3.73*
C	2.18	1.42	0.70	*2.12*	1.40	0.73	*2.13*
D	2.82	2.05	0.67	*2.72*	2.10	0.68	*2.78*
E	4.25	2.70	1.60	*4.30*	2.76	1.56	*4.32*
F	3.82	3.28	0.60	*3.88*	2.98	0.89	*3.87*
G		1.28	0.76	*2.04*	1.44	0.69	*2.13*
H		0.52	0.91	*1.43*	0.49	1.09	*1.58*
I	4.52	3.65	0.75	*4.40*	3.24	0.94	*4.18*
J	2.19	1.61	0.42	*2.03*	1.45	0.67	*2.12*
K	2.50	1.67	0.79	*2.46*	1.83	0.53	*2.36*
L	1.98	1.23	0.58	*1.81*	1.33	0.60	*1.93*
M	2.85	1.96	1.00	*2.96*	2.00	0.75	*2.75*
N	2.06	1.20	0.80	*2.00*	1.26	0.64	*1.90*
O	2.43	1.25	1.12	*2.37*	1.35	1.02	*2.37*
P	2.88	1.91	0.75	*2.66*	2.00	0.61	*2.61*
Q	2.83	1.55	1.00	*2.55*	1.60	0.93	*2.53*
R	2.28	1.26	0.70	*1.96*	1.38	0.62	*2.00*
S	1.83	1.09	0.69	*1.09*	1.19	0.63	*1.82*
T	2.39	1.00	1.00	*2.00*	1.08	1.01	*2.09*
U	1.95	0.97	0.90	*1.87*	0.90	—	—
V	1.62	0.82	0.70	*1.52*	0.82	0.77	*1.59*
W	1.38	0.58	0.72	*1.30*	0.60	0.68	*1.28*
X	1.91	1.13	0.70	*1.83*	1.06	0.88	*1.94*
Y	2.10	1.31	0.68	*1.99*	1.21	0.86	*2.10*
Z	1.61	0.77	0.70	*1.47*	0.80	0.76	*1.56*

carried by low-density lipoprotein fractions, which should be removed by spinning at 30,000 r.p.m. at density of serum before ultracentrifugation at salt density 1.063. Conversely, prior separation of these low-density fractions is not necessary with the heparin-phenol method, since it precipitates only beta lipoproteins. Therefore, in sera with total lipid content in a range of 500 to 1,200 mg. per cent, the heparin-phenol precipitation furnishes a satisfactory method for separation of beta lipoproteins. When total lipid content is higher than 1,200 mg. per cent preliminary dilution of the serum to concentration of between 600 to 900 mg. per cent is required for the method to be successful.

From the values of total cholesterol in the precipitate obtained from serum after addition of heparin-phenol, an indirect estimate of the concentration of beta lipoproteins in serum can be made, since about 31 per cent of the beta lipoproteins is cholesterol.[3] Further, from the amount of cholesterol recovered in the precipitate and in the supernatant, an estimation of the ratio of beta to alpha lipoproteins can be established. It may be assumed that the cholesterol, which is not precipitated from serum following addition of heparin and phenol, is present in the supernatant which contains alpha lipoprotein fractions.

SUMMARY

The ratio of cholesterol to phospholipid was similar in beta lipoproteins separated by ultracentrifugation or by heparin-phenol precipitation in 60 normal

and hyperlipemic human sera. Another study showed that the amount of cholesterol was also similar. In sera with total lipid content in a range of 500 to 1,200 mg. per cent, heparin-phenol precipitation furnishes a satisfactory method for separation of beta lipoproteins. In hyperlipemic sera in which total lipid values were greater than 1,200 mg. per cent, preliminary dilution to levels of total lipids between 600 and 900 mg. per cent was required for the method to be successful.

From the values of total cholesterol in the precipitate obtained from serum after addition of heparin and phenol, an indirect estimate of the concentration of beta lipoproteins in serum can be made.

We wish to thank Mr. S. Bárány and Mr. D. Yamashiro for their technical assistance.

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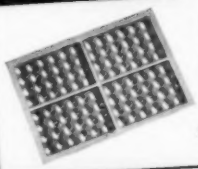

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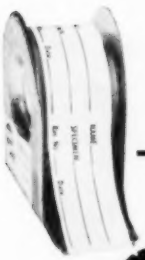

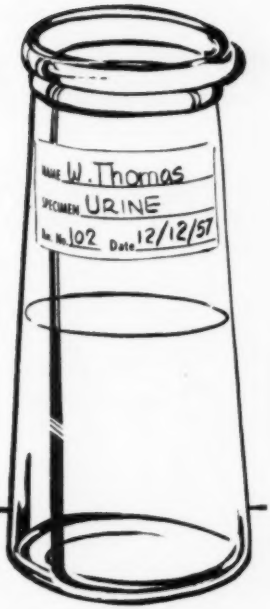

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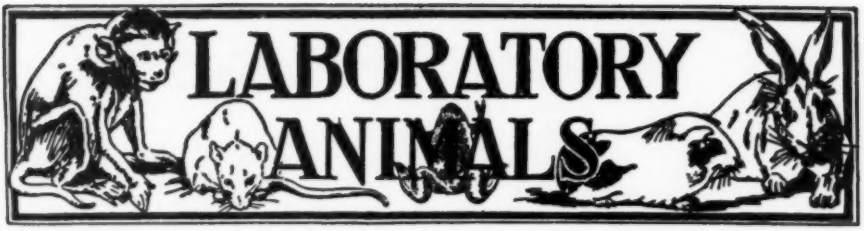

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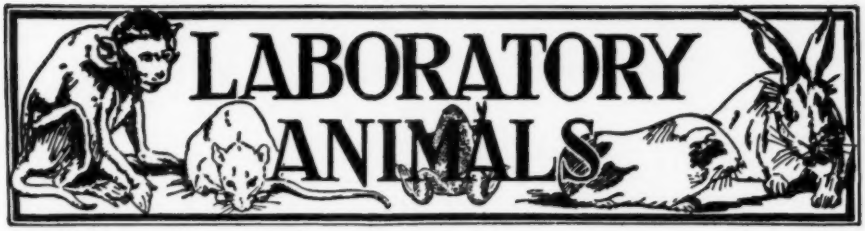

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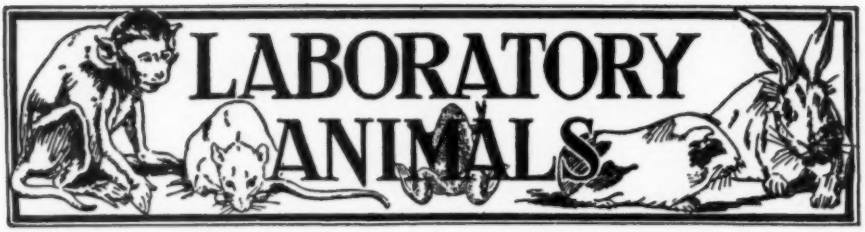

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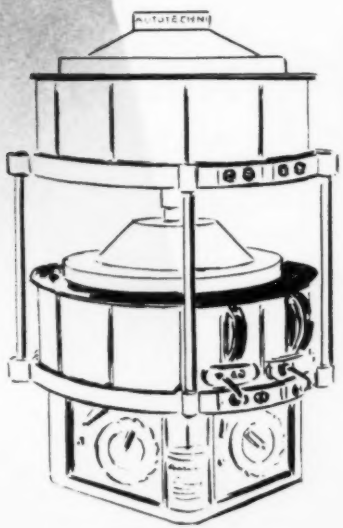

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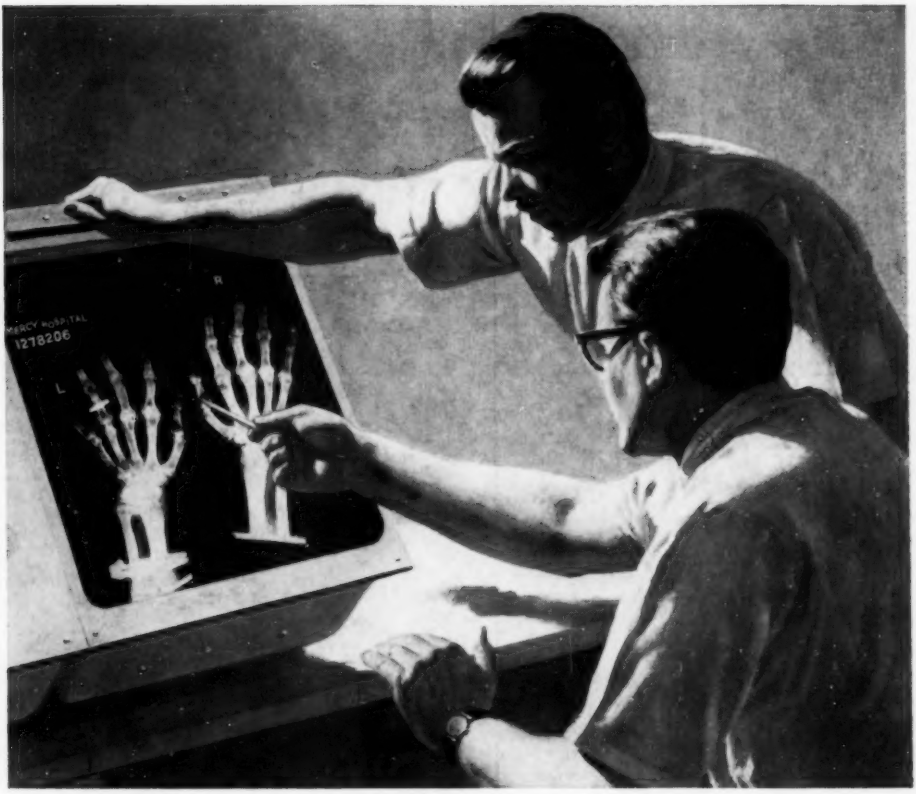

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